BIOGENIX INC. PVT. LTD.

Anti Phospholipid Screen

INTENDED USE

Anti Phospholipid Screen is an indirect solid phase immunoassay kit for the quantitative measurement of IgG and IgM class auto-antibodies directed against β2-glycoprotein mediated anionic phospholipids in human serum or plasma, including Cardiolipin, Phosphatidyl serine, Phosphatidyl inositol, Phosphatidic acid, Phosphatidyl choline, Lyso-phosphatidyl choline, Phosphatidyl ethanolamine. The assay is intended for in vitro diagnostic use only as an aid in the diagnosis of increased risk of thrombosis in patients with Systemic Lupus Eritematosus (SLE) or similar disorders.

Anti Phospholipid Screen kit is intended for laboratory use only.

1. CLINICAL SIGNIFICANCE

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 Eritematosus (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 µg / 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. PRINCIPLE

Anti Phospholipid Screen test is based on the binding of antibodies in human serum directed against the antigenic complex between anionic phospholipids (Cardiolipin, Phosphatidyl serine, Phosphatidyl inositol, Phosphatidic acid, Phosphatidyl choline, Lyso-phosphatidyl choline, Phosphatidyl ethanolamine) and β2-Glycoprotein; these complexes are coated on the microplate. Any antibody of IgG class or IgM class in calibrators, controls or prediluted patient samples binds to its respective antigen.After 60 minutes incubation, the microplate is washed with wash buffer for removing non-reactive serum components. An anti-human IgG conjugate solution (Conjugate IgG, reactive 4) or an anti-human IgM conjugate solution (Conjugate IgM, reactive 5) recognize IgG class or IgM class antibodies, respectively, bound to the immobilized antigens.After a 30 minutes incubation any excess enzyme conjugate which is not specifically bound is washed away with the wash buffer.A chromogenic substrate solution containing TMB is then dispensed into the wells. After a 15 minutes incubation the color development is stopped by adding the stop solution. The solutions

color change into yellow. The amount of color is directly proportional to the concentration of IgG or IgM antibodies present in the original sample.

The concentration of IgG or IgM antibodies in the original sample is calculated through a calibration curve.

3. REAGENTS, MATERIALS AND INSTRUMENTATION

3.1. Reagents and materials supplied in the kit

1. Calibrators (5 vials, 1,2 mL each) Phosphate buffer 0,1M, NaN_3 < 0,1%, human serum
CAL0**,** CAL1**,** CAL2**,** CAL3**,** CAL4

Controls (2 vials, 1,2 mL each, ready to use) Phosphate buffer 0,1M, NaN_3 < 0,1%, human serum

Positive Control**,** Negative Control

Sample Diluent (1 vial, 100 mL)Tampone fosfato 0,1 M NaN_3 < 0,1%

2. Conjugate IgG (1 vial, 15 mL) Anti h-IgG conjugate with horseradish peroxidise (HRP), BSA 0,1%, Proclin < 0,0015%
3. Conjugate IgM (1 vial, 15 mL) Anti h-IgM conjugate with horseradish peroxidise (HRP), BSA 0,1%, Proclin < 0,0015%
4. Coated Microplate (1 breakable microplate) Antigenic phospholipid and β2-Glicoprotein complexes coated on the microplate
5. TMB Substrate (1 vial, 15 mL) H_2O_2-TMB (0,26 g/L) *(avoid any skin contact)*
6. Stop Solution (1 vial, 15 mL) Sulphuric acid 0,15M *(avoid any skin contact)*
7. 10X Conc. Wash Solution (1 vial, 50 mL) Phosphate buffer 0,2M pH 7.4

3.2. Reagents necessary not supplied

Distilled water.

3.3. Auxiliary materials and instrumentation

Automatic dispenser.

Microplate reader (450 nm, 620-630 nm).

Notes

Store all reagents between 2÷8°C in the dark.

Open the bag of reagent 6 (Coated Microplate) only when it is at room temperature and close it immediately after use; once opened, it is stable until expiry date of the kit.

4. WARNINGS

- This kit is intended for in vitro use by professional persons only. Not for internal or external use in Humans or Animals.
- Use appropriate personal protective equipment while working with the reagents provided.
- Follow Good Laboratory Practice (GLP) for handling blood products.
- 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.
- All human source material used in the preparation of the reagents 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, Calibrators and Controls should be handled in the same manner as potentially infectious material.
- Some reagents contain small amounts of Sodium Azide (NaN_3) or Proclin 300^R 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/H_2O_2 to directed sunlight, metals or oxidants. Do not freeze the solution.

5. PRECAUTIONS

- Please adhere strictly to the sequence of pipetting steps provided in this protocol. The performance data represented here were obtained using specific reagents listed in this Instruction For Use.
- All reagents should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as 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 date printed on box and vials labels must be observed. Do not use any kit component beyond their 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.
  For this purpose, Biogenix supplies a separate decontamination reagent for cleaning needles.
- If you use automated equipment, the user has the 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. To improve the performance of the kit on automatic systems is recommended to increase the number of washes.
- 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, highly lipemeic or haemolysed should not be used in the assay.
- Plate readers measure vertically. Do not touch the bottom of the wells.

6. PROCEDURE

6.1. Preparation of Calibrators (C_0…C_4)

The Calibrators are ready to use and are mixed, so they have both IgG and IgM antibodies. The Calibrators have the following concentrations:

	C_0	C_1	C_2	C_3	C_4
AU/mL	0	5	10	20	80

Once opened, the Calibrators are stable 6 months at 2-8°C.

6.2. Sample Preparation

Either human serum or plasma samples can be used for the test execution. Test samples should be clear. Contamination by lipemia should be avoided, although it does not interfere with this assay. Specimens may be

refrigerated at 2-8°C for up to five days or stored at -20°C up to six months. Avoid repetitive freezing and thawing of serum samples. This may result in variable loss of autoantibody activity. Testing of heat-inactivated sera is not recommended.
All serum and plasma samples have to be diluted 1:100 with sample diluent; for example 10 µL of sample may be diluted with 990 µL of sample diluent.

The Controls are ready to use.

6.3. Wash Solution Preparation

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 the crystals are completely dissolved.

6.4. Procedure

- **Allow all reagents to reach room temperature (22-28°C) for at least 30 minutes.** At the end of the assay store immediately the reagents at 2-8°C: avoid long exposure to room temperature.
- Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C.
- To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.
- As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the calibration curve (C_0-C_4), two for each Control, two for each sample, one for Blank.

Reagents	Calibrator	Sample/ Controls	Blank
Calibrator C_0-C_4	**100 µL**		
Controls		**100 µL**	
Diluted Sample		**100 µL**	
Incubate 60 minutes at room temperature (22-28°C). Remove the content from each well, wash the wells 3 times with 300 µL of diluted wash solution. **Important note**: during each washing step, gently shake the plate for 5 seconds and remove excess solution by tapping the inverted plate on an absorbent paper towel. **Automatic washer**: if you use automated equipment, wash the wells at least 5 times.			
Conjugate (IgG or IgM)	**100 µL**	**100 µL**	
Incubate 30 minutes at room temperature (22-28°C). Remove the content from each well, wash the wells 3 times with 300 µL of diluted wash solution. **Washing**: follow the same indications of the previous point.			
TMB Substrate	**100 µL**	**100 µL**	**100 µL**
Incubate 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 a reference wavelength of 620-630 nm or against Blank within 5 minutes.

7. QUALITY CONTROL

- The anti-phopholipids Positive Control should be run with every batch of samples to ensure that all reagents and procedures perform properly.
- Because Positive Control is prediluted, it do not control for procedural methods associated with dilution of specimens.
- Additional suitable control sera may be prepared by aliquoting pooled human serum specimens and storing at < -20°C.
- In order for the test results to be considered valid, all of the criteria listed below must be met. If any of these are not met, the test should be considered invalid and the assay repeated:

- The Positive Control are intended to monitor for substantial reagent failure and they will not ensure precision at the assay cut-off.
- This test is only valid if the optical density at 450 nm for Positive Control as well as for the Calibrator (C_0-C_4) complies with the respective range indicated on the Quality Control Certificate enclosed to each test kit.

8. RESULTS

For Anti Phospholipid Screen kit 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 we recommend using a Lin-Log Plot.

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.

9. REFERENCE VALUES

In a normal range study with serum samples from healthy blood donors the following ranges have been established with the Anti Phospholipid Screen test:

	IgG (GPL AU/mL)	**IgM (MPL AU/mL)**
Normal	< 10	< 10
Elevated	≥ 10	≥ 10

Please pay attention to the fact that the determination of a range of expected values for a “normal” population in a given method is dependent on many factors, such as specificity and sensitivity of the method used and type of population under investigation. Therefore each laboratory should consider the range given by the Manufacurer as a general indication and produce their own range of expected values based on the indigenous population where the laboratory works.
Positive results should be verified concerning the entire clinical status of the patient. Also every decision for therapy should be taken individually. It is recommended that each laboratory establishes its own normal and pathological ranges of seric Ab-Anti-Phospholipid.

10. LIMITATIONS OF PROCEDURE

The presence of immune complexes or other immunoglobulin aggregates in the patient sample may cause an increased level of non-specific binding and produce false positives in this assay

11. PERFORMANCE AND CHARACTERISTICS

11.1. Precision and reproducibility

Precision and reproducibility are evaluated by eight reply of two positive samples by two different runs with two different lots. Dispensing and washing operations were performed manually by an operator.

The results in terms of standard deviation and coefficient of variation were below:

	IgG			
Sample	1		2	
	SD	CV%	SD	CV%
Intra-assay	1.03	5.9	1.31	7.4
Inter-assay	0.26	9.2	5.25	11.7
	IgM			
Sample	1		2	
	SD	CV%	SD	CV%
Intra-assay	0.61	7.6	1.97	5.9
Inter-assay	0.15	7.1	2.98	6.6

11.2. Sensitivity

The clinical sensitivity of Anti Phoshpolipids Screen IgG assay is 92,3%.
The clinical sensitivity of Anti Phoshpolipids Screen IgM assay is 68,8%.

11.3. Specificity

The clinical specificity of Anti Phoshpolipids Screen IgG assay is 84,6
The clinical specificity of Anti Phoshpolipids Screen IgM assay is > 99,9%.

11.4. Detection Limit

The lowest concentration that can be distinguished from Calibrator zero is 0.03 AU/mL for IgG and 0.16 AU/mL for IgM.

12. WASTE MANAGEMENT

Reagents must be disposed off in accordance with local regulations..

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