Name and Intended Use

Phosphatidyl Serine Ab is an indirect solid phase enzyme immunoassay (ELISA) for the quantitative measurement of IgG and IgM class autoantibodies against phosphatidyl serine in human serum or plasma.

Summary and Explanation of the Test

The first study of anti-phospholipid antibodies began in 1906, when Wasserman introduced a serological test for syphilis. In 1942, the active component was found to be a phospholipid, which was designated cardiolipin. In the 1950s it became clear that a number of people had positive tests for syphilis without any evidence of the disease. This phenomenon was referred to as the biological false positive serological test for syphilis. A high prevalence of autoimmune disorders, including systemic lupus erythematosus (SLE) and Sjögren´s syndrome occurred in this group of patients. The presence of circulating anticoagulants in patients with SLE was first documented in 1952 and was associated with increased risk of paradoxical thrombosis in 1963. The term lupus anticoagulant (LA), first used in 1972, is clearly a misnomer, because LA is more frequently encountered in patients without lupus and is associated with thrombosis rather than abnormal bleeding. During the last years it became clear that the optimal binding of anti-phospholipid antibodies is depending on a cofactor termed beta-2-glycoprotein I (apolipoprotein H) (β2GPI). β2GPI is a 50 kDa beta-2-globulin occurring in plasma at a level of 200 µg/ml. It has been found that beta-2-Glycoprotein I inhibits the intrinsic coagulation pathway and, therefore, it is involved in the regulation of blood coagulation. β2GPI is associated in vivo with negatively-charged substances, e.g. anionic phospholipids, heparin and lipoproteins. The phospholipid binding region is located on its fifth domain. Under the acronym "aPL" (anti-phospholipid antibodies) antibodies against negatively-charged phospholipids, such as CL (cardiolipin), LA (lupus anticoagulant), PS (phosphatidyl serine), PI (phosphatidyl inositol) and PA (phosphatidic acid) are summarised. Of these, cardiolipin is the phospholipid most commonly used as antigen to test for aPL by ELISA. Some antisera which bind cardiolipin-coated ELISA plates can also bind to plates coated with other negatively-charged phospholipids, such as phosphatidyl serine (PS), phosphatidyl inositol and phosphatidic acid (PA). Some investigators have suggested that the use of PS in place of cardiolipin in ELISA tests enables more specific diagnosis. These antigens are less commonly used and their additional use can improve the clinical sensitivity in patient samples with suspected APS (anti-phospholipidsyndrome), but they can't replace the measurement of autoantibodies against cardiolipin. The Scientific and Standardization Committee of the International Society on Thrombosis and Hemostasis has issued consensus criteria that may be used to help laboratory diagnosis. These criteria have been updated in 2006.

Principle of the Test

Highly purified phosphatidyl serine is bound to microwells saturated with beta-2-glycoprotein I. antibodies against these antigens, if present in diluted serum or plasma, bind to respective antigens. Washing of the microwells removes unspecific serum and plasma components. Horseradish peroxidase (HRP) conjugated anti-human IgG and IgM immunologically detects the bound patient antibodies forming a conjugate/antibody/antigen complex. Washing of the microwells removes unbound conjugate. An enzyme substrate in the presence of bound conjugate hydrolyses to form a blue colour. The addition of an acid stops the reaction forming a yellow end-product. The intensity of this yellow colour is measured photometrically at 450 nm. The amount of colour is directly proportional to the concentration of IgG resp. IgM antibodies present in the original sample.

Warnings and Precautions

1. All reagents of this kit are strictly intended for in vitro diagnostic use only.
2. Do not interchange kit components from different lots.
3. Components containing human serum were tested and found negative for HBsAg, HCV, HIV1 and HIV2 by FDA approved methods. No test can guarantee the absence of HBsAg, HCV, HIV1 or HIV2, and so all human serum based reagents in this kit must be handled as though capable of transmitting infection.
4. Avoid contact with the TMB (3,3´,5,5´-Tetramethyl-benzidine). If TMB comes into contact with skin, wash thoroughly with water and soap.
5. Avoid contact with the Stop Solution which contains hydrochloric acid (1 M). If it comes into contact with skin, wash thoroughly with water and seek medical attention.
6. Some kit components (i.e. Controls, Sample buffer and Buffered Wash Solution) contain Sodium Azide as preservative. Sodium Azide (NaN3) is highly toxic and reactive in pure form. At the product concentrations (0.09%), though not hazardous. Despite the classification as nonhazardous, we strongly recommend using prudent laboratory practices (see 8., 9., 10.)
7. Some kit components contain Proclin 300 as preservative. When disposing reagents containing Proclin 300, flush drains with copious amounts of water to dilute the components below active levels.
8. Wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.
9. Do not pipette by mouth.
10. Do not Eat, Drink, Smoke or Apply Makeup in areas where specimens or kit reagents are handled.
11. Avoid contact between the buffered Peroxide Solution and easily oxidized materials; extreme temperature may initiate spontaneous combustion.

Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. During handling of all kit reagents, controls and serum samples observe the existing legal regulations.

Contents of the Kit

Package size	96 determ.
Qty.1	Divisible microplate consisting of 12 modules of 8 wells each, coated with highly purified phosphatidyl serin and saturated with beta-2-Glycoprotein I. Ready to use.
6 vials, 1.5 ml each	combined Calibrators with IgG and IgM class anti-phospholipid antibodies (AF) in a serum/buffer matrix (PBS, BSA, NaN3 <0.1% (w/w)) containing: IgG: 0; 6.3; 12.5; 25; 50; and 100 GPL U/ml and IgM: 0; 6.3; 12.5; 25; 50; 100 MPL U/ml. Ready to use.
2 vials, 1,5 ml each	Anti-C1q controls in a serum/buffer matrix (PBS, BSA, NaN3 <0.1% (w/w)) positive (1) and negative (2), for the respective concentrations see the enclosed package insert. Ready to use.
2 vials, 1,5 ml each	Anti-Phospholipid Controls in a serum/buffer matrix (PBS, BSA, NaN3 <0.1% (w/w)) positive (1) and negative (2), for the respective concentrations see the enclosed package insert. Ready to use.
1 vial, 20 ml	Sample buffer (Tris, NaN3 <0,1% (w/w)), yellow, concentrate (5x)
1 vial, 15 ml	Enzyme conjugate solution (PBS, PROCLIN 300 <0,5% (v/v)), (light red) containing polyclonal rabbit anti-human IgA; labelled with horseradish peroxidase. Ready to use
1 vial, 15 ml	TMB substrate solution. Ready to use
1 vial, 15 ml	Stop solution (1 M hydrochloric acid). Ready to use
1 vial, 20 ml	Wash solution (PBS, NaN3 <0,1% (w/w)), concentrate (50x)

Storage and Stability

1. Store the kit at 2-8℃.
2. Keep microplate wells sealed in a dry bag with desiccants.
3. The reagents are stable until expiration of the kit.
4. Do not expose test reagents to heat, sun or strong light during storage and usage.
5. Diluted sample buffer and wash buffer are stable for at least 30 days when stored at 2-8℃.

Materials Required

Equipment

- ✓ Microplate reader capable of endpoint measurements at 450 nm
- ✓ Multi-Channel Dispenser or repeatable pipet for 100 µl
- ✓ Vortex mixer

- ✓ Pipets for 10 µl, 100 µl and 1000 µl
- ✓ Laboratory timing device
- ✓ data reduction software

Preparation of reagents

- ✓ distilled or deionized water
- ✓ graduated cylinder for 100 and 1000 ml
- ✓ plastic container for storage of the wash solution

Specimen Collection, Storage and Handling

1. Collect whole blood specimens using acceptable medical techniques to avoid hemolysis
2. Allow blood to clot and separate the serum by centrifugation
3. Test serum should be clear and non-hemolyzed. Contamination by hemolysis or lipemia is best avoided, but does not interfere with this assay.
4. Specimens may be refrigerated at 2-8℃ for up to five days or stored at –20℃ up to six months.
5. Avoid repetitive freezing and thawing of serum samples. This may result in variable loss of autoantibody activity
6. Testing of heat-inactivated sera is not recommended

Procedural Notes

1. Do not use kit components beyond their expiration dates.
2. Do not interchange kit components from different lots.
3. All materials must be at room temperature (20-28 ℃).
4. Have all reagents and samples ready before start of the assay. Once started, the test must be performed without interruption to get the most reliable and consistent results.
5. Perform the assay steps only in the order indicated.
6. Always use fresh sample dilutions.
7. Pipette all reagents and samples into the bottom of the wells.
8. To avoid carryover contamination, change the tip between samples and different kit controls.
9. It is important to wash microwells thoroughly and remove the last droplets of wash buffer to achieve best results.
10. All incubation steps must be accurately timed.
11. Control sera or pools should routinely be assayed as unknowns to check performance of the reagents and the assay.
12. Do not re-use microplate wells.

For all controls, the respective concentrations are provided on the labels of each vial. Using these concentrations a calibration curve may be calculated to read off the patient results semiquantitatively.

Preparation of Reagents

Preparation of sample buffer

Dilute the contents of each vial of the sample buffer concentrate (5x) with distilled or deionized water to a final volume of 100 ml prior to use. Store refrigerated: stable at 2-8℃ for at least 30 days after preparation or until the expiration date printed on the label.

Preparation of wash solution

Dilute the contents of each vial of the buffered wash solution concentrate (50x) with distilled or deionized water to a final volume of 1000 ml prior to use. Store refrigerated: stable at 2-8℃ for at least 30 days after preparation or until the expiration date printed on the label.

Sample preparation

Dilute all patient samples 1:100 with sample buffer before assay. Therefore combine 10 µl of sample with 990 µl of sample buffer in a polystyrene tube. Mix well. Controls are ready to use and need not be diluted.

Test Procedure

1. Prepare a sufficient number of microplate modules to accommodate controls and prediluted patient samples.
2. Pipette **100 µl** of calibrators, controls and prediluted patient samples in duplicate into the wells.
3. Incubate for 30 minutes at room temperature (20-28 ℃)

	1	2	3	4	5	6
A	SA	SE	P1	P5		
B	SA	SE	P1	P5		
C	SB	SF	P2	P6		
D	SB	SF	P2	P..		
E	SC	C1	P3	P..		
F	SC	C1	P3			
G	SD	C2	P4			
H	SD	C2	P4			

SA-SF : standard A to F

P1, P2...C : patient sample 1, 2...

C1 : positive control

C2 : negative control

4. Discard the contents of the microwells and wash 3 times with **300 µl** of wash solution.
5. Dispense **100 µl** of enzyme conjugate into each well.

6. Incubate for 15 minutes at room temperature.
7. Discard the contents of the microwells and wash 3 times with **300 µl** of wash solution.
8. Dispense **100 µl** of TMB substrate solution into each well.
9. Incubate for 15 minutes at room temperature.
10. Add **100 µl** of stop solution to each well of the modules and incubate for 5 minutes at room temperature.
11. Read the optical density at 450 nm and calculate the results. Bi-chromatic measurement with a reference at 600-690 nm is recommended.

The developed colour is stable for at least 30 minutes. Read optical densities during this time.

Interpretation of Results

Quality Control

This test is only valid if the optical density at 450 nm for Positive Control (1) and Negative Control (2) as well as for the Calibrator A and F complies with the respective range indicated on the Quality Control Certificate enclosed to each test kit! If any of these criteria is not met, the results are invalid and the test should be repeated.

Calculation of results

For Phosphatidyl Serine Ab a 4-Parameter-Fit with lin-log coordinates for optical density and concentration is the data reduction method of choice.

Recommended 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.

Calculation example

The figures below show typical results for anti-Phospholipid IgG and IgM. These data are intended for illustration only and should not be used to calculate results from another run.

Calibrators										
Anti-PA	No	Position	OD 1	OD 2	Mean	Conc. 1	Conc. 2	Mean	decl. Conc.	CV %
IgG	STA	A1/B1	0.085	0.091	0.088	0	0.3	0.2	0	5
IgG	STB	C1/D1	0.277	0.222	0.225	6	5.8	5.9	6.3	2
IgG	STC	E1/F1	0.37	0.376	0.373	12	12.3	12.2	12.5	1
IgG	STD	G1/H1	0.687	0.703	0.695	26	27	27	25	2
IgG	STE	A2/B2	1.109	1.113	1.111	48	48	48	50	0
IgG	STF	C2/D2	1.911	1.881	1.896	102	100	101	100	1
IgM	STA	A7/B7	0.031	0.033	0.032	0	0.1	0	0	4
IgM	STB	C7/D7	0.239	0.249	0.244	6.1	6.3	6.2	6.3	3
IgM	STC	E7/F7	0.458	0.465	0.462	12.5	12.7	12.6	12.5	1
IgM	STD	G7/H7	0.791	0.826	0.809	24	26	25	25	3
IgM	STE	A8/B8	1.289	1.299	1.294	50	51	50	50	1
IgM	STF	C8/D8	1.791	1.784	1.788	101	99	100	100	0

Interpretation of results

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

	anti-β2-Glycoprotein I-Ab	
	IgG [GPL U/ml]	IgM [MPLU/ml]
normal :	< 10	< 10
elevated :	≥10	≥10

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 serum anti-phospholipid.

Performance Characteristics

Specificity

The microplate is coated with highly purified Phosphatidyl Serine and saturated with human beta-2-glycoprotein I. Special coating processes, developed by the manufacturer guarantee for the native immunogenic structure of the phospholipids after immobilization on the solid phase. The ELISA kit is specific for autoantibodies directed against the respective phospholipid or the complex of the negatively-charged phospholipid and beta-2-Glycoprotein I. No cross reactivity was observed to anti-DNA antibodies and those types of antibodies occurring in syphilis.

Calibration

The assay system is calibrated against the internationally recognised reference sera from E. N. Harris, Louisville, USA, IRP 97/656 and HCAL/EY2C9 since no other international standards are available.

Limitations of Procedure

The Phosphatidyl Serine Ab ELISA is a diagnostic aid. A definite clinical diagnosis should not be based on the results of a single test, but should be made by the physician after all clinical and laboratory findings have been evaluated.

Interfering Substances

No interference has been observed with haemolytic (up to 1000 mg/dL), lipemic (up to 3 g/dL triglycerides) or bilirubin (up to 40 mg/dL) containing sera. Nor have any interfering effects been observed with the use of anticoagulants. However for practical reasons it is recommended that grossly hemolysed or lipemic samples should be avoided.

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