Anti-Phosphatidylserine ELISA (IgA)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG-CLASS	SUBSTRATE	FORMAT
EA 162a-9601 A	Phosphatidylserine	IgA	Ag-coated microplate wells	96 x 01 (96)

Indication: Anti-phospholipid syndrome.

Principles of the test: The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human autoantibodies of the IgA class against phosphatidylserine in serum or plasma. The test kit contains microtiter strips each with 8 break-off reagent wells coated with phosphatidylserine. In the first reaction step, diluted patient samples are incubated with the wells. In many cases, antibodies to phosphatidylserine rely on a plasma protein (β2-glycoprotein I) as a cofactor for antigen recognition. The reaction buffer must therefore contain this cofactor. In the case of positive samples, the specific IgA antibodies (also IgG and IgM) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgA (enzyme conjugate) catalysing a colour reaction. The intensity of the formed colour is proportional to the concentration of antibodies to phosphatidylserine.

Contents of the test kit:

Component	Colour	Format	Symbol
1. Microplate wells, coated with antigens: 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use	---	12 x 8	STRIPS
2. Calibrator 1 120 RU/ml (IgA, human), ready for use	dark red	1 x 2.0 ml	CAL 1
3. Calibrator 2 12 RU/ml (IgA, human), ready for use	red	1 x 2.0 ml	CAL 2
4. Calibrator 3 2 RU/ml (IgA, human), ready for use	light red	1 x 2.0 ml	CAL 3
5. Positive control (IgA, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
6. Negative control (IgA, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
7. Enzyme conjugate peroxidase-labelled anti-human IgA (rabbit), 10x concentrate	orange	1 x 1.5 ml	CONJUGATE 10x
8. Sample buffer contains ß2-glycoprotein I, ready for use	yellow	1 x 100 ml	SAMPLE BUFFER
9. Wash buffer 10x concentrate.	colourless	1 x 100 ml	WASH BUFFER 10x
10. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
11. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
12. Test instruction	---	1 booklet	
13. Quality control certificate	---	1 protocol	

LOT Lot description — CE — Storage temperature

IVD In vitro diagnostics — Unopened usable until

Storage and stability: The test kit has to be stored at a temperature between +2°C to +8°C. The **calibrators and positive control** must be **stored in aliquots at -20°C**. Unopened, all test kit components are stable until the indicated expiry date.

Preparation and stability of the reagents

Note: All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. After first use, the reagents are stable until the indicated expiry date if stored at +2°C to +8°C and protected from contamination, unless stated otherwise below.

- **Coated wells:** Ready for use. Tear open the resealable protective wrapping of the microplate at the recesses above the grip seam. Do not open until the microplate has reached room temperature to prevent the individual strips from moistening. Immediately replace the remaining wells of a partly used microplate in the protective wrapping and tightly seal with the integrated grip seam (Do not remove the desiccant bag).
  Once the protective wrapping has been opened for the first time, the wells coated with antigens can be stored in a dry place and at a temperature between +2°C and +8°C for 4 months.

- **Calibrators and controls:** Ready for use. The reagents must be mixed thoroughly before use.

- **Enzyme conjugate:** The enzyme conjugate is a 10x concentrate and should be mixed thoroughly before use. The amount required should be removed from the bottle using a clean pipette and diluted 1:10 with sample buffer. For example, dilute 0.1 ml enzyme conjugate with 0.9 ml buffer for 8 microplate wells. The diluted ready to use enzyme conjugate is to be used within 4 hours.

- **Sample buffer:** Ready for use.

- **Wash buffer:** The wash buffer is a 10x concentrate. If crystallization occurs in the concentrated buffer, warm it to 37°C and mix well before diluting. The quantity required should be removed from the bottle using a clean pipette and diluted with deionized or distilled water (1 part reagent plus 9 parts distilled water).
  For example: For 1 microplate strip, 5 ml concentrate plus 45 ml water.
  The working strength wash buffer is stable for 4 weeks when stored at +2°C to +8°C and handled properly.

- **Chromogen/substrate solution:** Ready for use. Close the bottle immediately after use, as the contents are sensitive to light. The chromogen/substrate solution must be clear on use. Do not use the solution if it is blue coloured.

- **Stop solution:** Ready for use.

Warning: Calibration and controls used have been tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2 using enzyme immunoassays and indirect immunofluorescence methods. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the toxic agent sodium azide. Avoid skin contact.

Waste disposal: Patient samples, calibrators, controls and incubated microplate strips shoud be handled as infectious waste. All reagents must be disposed of in accordance with local disposal regulations.

Preparation and stability of the patient samples

Samples: Human serum or EDTA, heparin or citrate plasma.

Stability: Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Diluted samples should be incubated within one working day.

Sample dilution: Patient samples are diluted **1:201** in sample buffer. For example: dilute 5 µl serum in 1.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

NOTE: Calibrators and controls are prediluted and ready for use, do not dilute them.

Incubation

For **semiquantative analysis** incubate **calibrator 2** along with the positive and negative controls and patient samples. For **quantitative analysis** incubate **calibrators 1, 2 and 3** along with the positive and negative controls and patient samples.

Sample incubation: (1st step)
Transfer 100 µl of the calibrators, positive and negative controls or diluted patient samples into the individual microplate wells according to the pipetting protocol. Incubate for **30 minutes** at room temperature (+18°C to +25°C).

Washing:
Manual: Empty the wells and subsequently wash 3 times using 300 µl of working strength wash buffer for each wash.
Automatic: Wash reagent wells 3 times with 450 µl working strength wash buffer (program setting: e.g. TECAN Columbus Washer “Overflow Modus”).

Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual and automated tests), thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.

Note: Residual liquid (> 10 µl) remaining in the reagent wells after washing can interfere with the substrate and lead to false low extinction values. Insufficient washing (e.g., less than 3 wash cycles, too small wash buffer volumes, or too short residence times) can lead to false high extinction values.
Free positions on the microplate strip should be filled with blank wells of the same plate format as that of the parameter to be investigated.

Conjugate incubation: (2nd step)
Pipette 100 µl of enzyme conjugate (peroxidase-labelled anti-human IgA) into each of the microplate wells. Incubate for **30 minutes** at room temperature (+18°C to +25°C).

Washing:
Empty the wells. Wash as described above.

Substrate incubation: (3rd step)
Pipette 100 µl of chromogen/substrate solution into each of the microplate wells. Incubate for **15 minutes** at room temperature (+18°C to +25°C) protect from direct sunlight.

Stopping:
Pipette 100 µl of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.

Measurement:
Photometric measurement of the colour intensity should be made at a **wavelength of 450 nm** and a reference wavelength between 620 nm and 650 nm **within 30 minutes of adding the stop solution.** Prior to measuring, slightly shake the micro-plate to ensure a homogeneous distribution of the solution.

Pipetting protocol

	1	2	3	4	5	6	7	8	9	10	11	12
A	**C 2**	**P 6**	**P 14**	**P 22**			**C 1**	**P 4**	**P 12**	**P 20**		
B	**pos.**	**P 7**	**P 15**	**P 23**			**C 2**	**P 5**	**P 13**	**P 21**		
C	**neg.**	**P 8**	**P 16**	**P 24**			**C 3**	**P 6**	**P 14**	**P 22**		
D	**P 1**	**P 9**	**P 17**				**pos.**	**P 7**	**P 15**	**P 23**		
E	**P 2**	**P 10**	**P 18**				**neg.**	**P 8**	**P 16**	**P 24**		
F	**P 3**	**P 11**	**P 19**				**P 1**	**P 9**	**P 17**			
G	**P 4**	**P 12**	**P 20**				**P 2**	**P 10**	**P 18**			
H	**P 5**	**P 13**	**P 21**				**P 3**	**P 11**	**P 19**			

The pipetting protocol for microtiter strips 1-4 is an example for the **qualitative/semiquantitative analysis** of 24 patient samples (P 1 to P 24).
The pipetting protocol for microtiter strips 7-10 is an example for the **quantitative analysis** of 24 patient samples (P 1 to P 24).

The calibrators (C 1 to C 3), the positive (pos.) and negative (neg.) controls, and the patient samples have each been incubated in one well. The reliability of the ELISA test can be improved by duplicate determinations for each sample.
The wells can be broken off individually from the strips. This makes it possible to adjust the number of test substrates used to the number of samples to be examined and minimizes reagent wastage.
Both positive and negative controls serve as internal controls for the reliability of the test procedure. They should be assayed with each test run.

Calculation of results

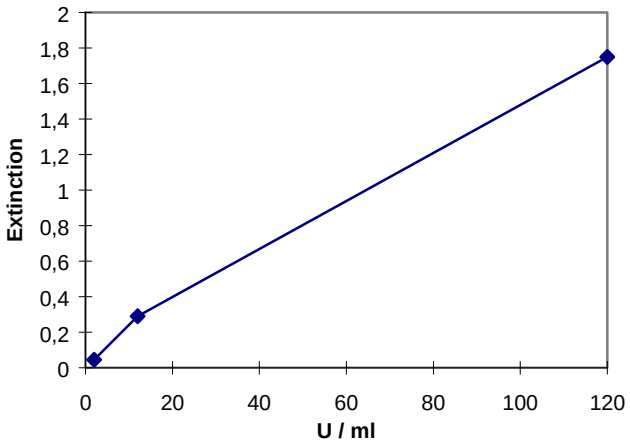
Qualitative/semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of calibrator 2. Calculate the ratio according to the following formula:

$$\frac{\text{Extinction of the control or patient sample}}{\text{Extinction of calibrator 2}} = \text{Ratio}$$

EUROIMMUN recommends interpreting results as follows:

Ratio <1.0: negative
Ratio ≥1.0: positive

Quantitative: The standard curve from which the concentration of antibodies in the serum samples can be taken is obtained by point-to-point plotting of the extinction values measured for the 3 calibration sera against the corresponding units (linear/linear). Use “point-to-point” plotting for calculation of the standard curve by computer. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of antibody concentrations in patient samples.



If the extinction of a serum sample lies above the value of calibrator 1 (120 RU/ml). The result shold be given as “>120 RU/ml“. It is recommended that the sample be re-tested at a dilution of 1:800. The result in RU/ml read from the calibration curve for this sample must then be multiplied by a factor of 4.

The upper limit of the normal range **(cut-off)** recommended by EUROIMMUN is 12 relative units (RU)/ml. EUROIMMUN recommends interpreting results as follows:

<12 RU/ml:	**negative**
≥12 RU/ml:	**positive**

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another the sample should be retested.

For diagnosis, the clinical symptoms of the patient should always be taken into account along with the serological results.

Test characteristics

Calibration: As no international reference serum exists for the measurement of antibodies against phosphatidylserine, the calibration is performed in relative units (RU)/ml.

For every group of tests performed, the extinction values of the calibrators and the relative units and/or ratio determined for the positive and negative controls must lie within the limits stated for the relevant test kit lot. A quality control certificate containing these reference values is included. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

The activity of the enzyme used is temperature-dependent and the extinction values may vary if a thermostat is not used. The higher the room temperature (+18°C to +25°C) during substrate incubation, the greater will be the extinction values. Corresponding variations apply also to the incubation times. However, the calibrators are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antigen: The microplate wells were coated with purified phosphatidylserine isolated from bovine brain. Phosphatidylserine is a negatively charged phospholipid which is frequently located in cellular membranes. Phospholipids consist of a phosphoric acid that is esterified on one side with a glycerol derivative and on the other with serine, choline, ethanolamine, inositol or glycerol. The glycerol derivative contains two fatty acids which can vary in length and in the number of their double bonds. A glycerol derivative of this kind forms a phosphatide acid with the esterified phosphoric acid.

Antibodies against phosphatidylserine are probably a subtype of a class of closely related antibodies to anionic phospholipids (e.g., cardiolipin, phosphatidylserine, phosphatidylinositol) which differ in their affinities. A subpopulation of antibodies against anionic phospholipids relies on a plasma protein (β2-glycoprotein I, GPI) as a cofactor for antigen recognition. It is presently not clear whether this antibody population only recognizes epitopes of the GPI or also epitopes of the phosphatidylserin. GPI is known to interact only with anionic but not with neutral phospholipids (e.g. phosphatidyethanolamine).

Linearity: The linearity of the Anti-Phosphatidylserine ELISA (IgA) was determined by assaying 4 serial dilutions of different patient samples. The coefficient of determination R^2 for all sera was > 0.95. The Anti-Phosphatidylserine ELISA (IgA) is linear at least in the tested concentration range (7 RU/ml to 114 RU/ml).

Detection limit: The lower detection limit is defined as the mean value of an analyte-free sample plus three times the standard deviation and is the smallest detectable antibody titer. The lower detection limit of the Anti-Phosphatidylserine ELISA (IgA) is 1.7 RU/ml.

Cross reactivity: No cross reactions with other autoantibodies have been found. An exception is antibodies against other negatively charged phospholipids (e.g. cardiolipin) which exhibit cross reactivity as a result of their structural homologies.

Interference: Haemolytic, lipaemic and icteric samples showed no influence on the result up to a concentration of 10 mg/ml for hemoglobin, 20 mg/ml for triglycerides and 0.4 mg/ml for bilirubin in this ELISA.

Reproducibility: The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation (CV) using 3 sera. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 4 determinations performed in 6 different test runs.

Intra-assay variation, n = 20		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	37	3.9
2	60	6.9
3	65	4.6

Inter-assay variation, n = 4 x 6		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	44	8.6
2	64	5.0
3	66	4.8

Prevalence and specificity: The prevalence anti-phosphatidylserine antibodies (IgA) in a panel of 21 APS patients was 14%. The specificity in a control panel of patients with HIV, HBV or HCV (n = 247), healthy pregnant women (n = 200) and healthy blood donors (n = 200) was 100%.

Reference range: The levels of the anti-phosphatidylserine antibodies (IgA) were analyzed with this EUROIMMUN ELISA in 200 healthy blood donors. With a cut-off of 12 RU/ml, all blood donors were anti-phosphatidylserine negative (IgA).

Clinical significance

The clinical complications associated with the occurence of anti-cardiolipin antibodies are collectively known as **anti-phospholipid syndrome**: venous and arterial thrombosis, thrombocytopenia, spontaneous abortion, still births and premature births; involvement of the central nervous system (from headaches to cerebral thrombosis); early signs of bone necrosis; pulmonary hypertonia.

Antibodies against cardiolipin are found in ca. 50% of cases of disseminated lupus erythematosus and in ca. 5% - 40% of patients with other systemic autoimmune disorders (rheumatoid arthritis, scleroderma, Sjögren's syndrome and Sharp syndrome, among others). Patients with antibodies against cardiolipin are considered to be at risk for the development of venous and arterial thromboses (with high levels of antibodies to cardiolipin the predictability for this risk parameters is about 80%).

Spontaneous abortion, still births and premature births are continuously being observed in cases where antibodies to cardiolipin can be demonstrated, independently of whether symptoms of an autoimmune condition are present. However, patients with disseminated lupus erythematodes are particular prone to the described complications in pregnancy (up to 77% of cases). The causes being discussed include infarcts in the placenta as a result of venous thromboses.

High titres of anti-cardiolipin antibodies after survival of a myocardio- or cerebral infarct can be an indication of an elevated risk of further vascular complications as well as being a means of interpreting the clinical condition and prognosis of survival after such an infarct.

Autoantibodies to cardiolipin can belong to the immunoglobulin subclasses IgA, IgG or IgM. Of greatest diagnostic relevance are high IgG concentrations, but a higher number of patients with cardiolipin antibodies can be detected by the additional determination of IgA and IgM levels. Furthermore, evidence exists that there is a strong correlation between high concentrations of IgG cardiolipin antibodies with thrombocytopenia and between high concentrations of IgM cardiolipin antibodies and haemolytic anaemia.

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