Anti-Cardiolipin ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EA 1621-9601 G	Cardiolipin (β2GP1 as cofactor)	IgG	Ag-coated microplate wells	96 x 01 (96)

Indication: The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human antibodies of the immunoglobulin class IgG against cardiolipin in serum or plasma for the diagnosis of antiphospholipid syndrome (APS).

Application: According to the official classification criteria for anti-phospholipid syndrome, antibodies against cardiolipin or β2 glycoprotein 1 (Ig classes G or M) or a positive lupus anticoagulant test (LA test) are the admissible serological parameters which allow for APS diagnosis. In the criteria's extension from 2012, it is recommended to also investigate antibodies of class IgA, alongside IgG and IgM determination in the case of negative ACA or anti-β2GP1 findings. Therefore, depending on the diagnostic strategy, also test systems for the determination of all immunoglobulin classes (IgAGM) are suitable.

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

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 PL-IgG-units/ml (human), ready for use	dark red	1 x 2.0 ml	CAL 1
3. Calibrator 2 12 PL-IgG-units/ml (human), ready for use	red	1 x 2.0 ml	CAL 2
4. Calibrator 3 2 PL-IgG-units/ml (human), ready for use	light red	1 x 2.0 ml	CAL 3
5. Positive control (IgG, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
6. Negative control (IgG, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
7. Enzyme conjugate peroxidase-labelled anti-human IgG (rabbit), 10x concentrate	green	1 x 1.5 ml	CONJUGATE
8. Sample buffer contains β2-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
IVD In vitro diagnostic medical device
CE
Storage temperature
Unopened usable until

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: For 1 microplate strip, 0.1 ml concentrate plus 0.9 ml sample buffer.
  The working strength diluted enzyme conjugate is to be used within 4 hours.

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

- **Wash buffer:** The wash buffer is a 10x concentrate. If crystallisation 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 deionised 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.

Storage and stability: The test kit has to be stored at a temperature between +2°C to +8°C. Do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

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

Warning: The calibrators and controls of human origin have tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the agent sodium azide in a non-declarable concentration. Avoid skin contact.

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

NOTE: The 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.

(Partly) manual test performance

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 the reagent wells 3 times with 450 µl of working strength wash buffer (program setting: e.g. TECAN Columbus Washer "Overflow Mode").

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) 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 IgG) 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 microplate to ensure a homogeneous distribution of the solution.

Test performance using fully automated analysis devices

Sample dilution and test performance are carried out fully automatically using an analysis device. The incubation conditions programmed in the respective software authorised by EUROIMMUN may deviate slightly from the specifications given in the ELISA test instruction. However, these conditions were validated in respect of the combination of the EUROIMMUN Analyzer I, Analyzer I-2P or the DSX from Dynex and this EUROIMMUN ELISA. Validation documents are available on enquiry.
Automated test performance using other fully automated, open-system analysis devices is possible. However, the combination should be validated by the user.

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

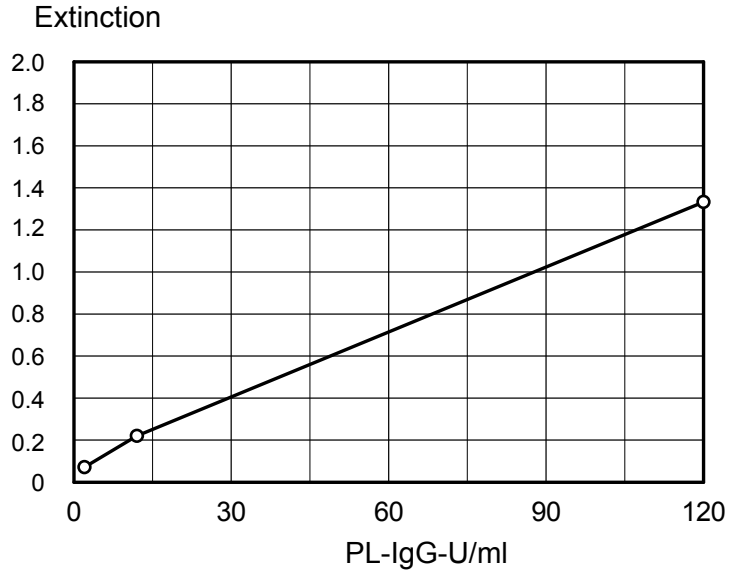
Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of the calibrator 2. Calculate the ratio according to the following formula:

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

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 patient 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 for a patient sample lies above the value of calibrator 1 (120 PL-IgG-U/ml), the result should be reported as ">120 PL-IgG-U/ml". It is recommended that the sample be retested at a dilution of e.g. 1:800. The result in U/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 phospholipid-IgG-units/ml (PL-IgG-U/ml). EUROIMMUN recommends interpreting results as follows:

<12 PL-IgG-U/ml	**negative**
≥12 PL-IgG-U/ml	**positive**

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another, EUROIMMUN recommends retesting the samples.

For diagnosis, the clinical picture of the patient always needs to be taken into account along with the serological findings.

Test characteristics

Calibration: The calibration of the controls is performed in PL-IgG-units using an international human standard serum (Louisville APL Diagnostics, USA). 1 PL-IgG-unit is defined as the cardiolipin binding activity of 1 µg/ml of an affinity purified IgG anti-cardiolipin preparation from a standard serum (Harris et al., 1987).

For every group of tests performed, the extinction values of the calibrators and the 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 binding activity of the antibodies and the activity of the enzyme used are temperature-dependent. It is therefore recommended using a thermostat in all three incubation steps. The higher the room temperature (+18°C to +25°C) during the incubation steps, 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: Cardiolipin is a negatively charged phospholipid which is located in high concentrations in the inner mitochondrial membrane (of nine known mitochondrial antigen types it is classified as M1). Phospholipids consist of a phosphoric acid esterified to, on the one hand, a glycerol derivative, and on the other hand, serine, choline, ethanolamine, inositol or glycerol. The glycerol derivative contains two fatty acids with double bindings of varying number and length. The glycerol derivative together with the esterified phosphoric acid form a phosphatidic acid. In cardiolipin two phosphatidic acids are linked to a further glycerol.

Antibodies against cardiolipin 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 cardiolipin (ca. 75%) 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 recognises epitopes of the GPI or also epitopes of the cardiolipin. GPI is known to interact only with anionic but not with neutral phospholipids (e.g. phosphatidyethanolamine).

Linearity: The linearity of the Anti-Cardiolipin ELISA (IgG) was determined by assaying at least 4 serial dilutions of different patient samples. The Anti-Cardiolipin ELISA (IgG) is linear at least in the tested concentration range (4 PL-IgG-U/ml to 98 PL-IgG-U/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-Cardiolipin ELISA (IgG) is 0.8 PL-IgG-U/ml.

Cross reactivity: Owing to the distinct structural homology of the phospholipids, antibodies to cardiolipin will cross-react with other phospholipids (phosphatidylserine, -inositol, -glycerine, -ethanolamine and -choline). No other cross-reactions are known.

Interference: Haemolytic, lipaemic and icteric samples showed no influence at the result up to a concentration of 10 mg/ml for haemoglobin, 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 samples. 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		
Sample	**Mean value (PL-IgG-U/ml)**	**CV (%)**
1	25	9.6
2	54	5.7
3	78	5.9

Inter-assay variation, n = 4 x 6		
Sample	**Mean value (PL-IgG-U/ml)**	**CV (%)**
1	26	10.4
2	54	11.4
3	79	8.5

Prevalence and specificity: The prevalence anti-cardiolipin antibodies (IgG) in a panel of 21 APS patients was 67%. 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 = 406) was 100%.

Reference range: The levels of the anti-cardiolipin antibodies (IgG) were analysed with this EUROIMMUN ELISA in 400 healthy blood donors. With a cut-off of 12 PL-IgG-U/ml, all blood donors were anti-cardiolipin negative (IgG).

Clinical significance

Anti-cardiolipin antibodies (ACA, ACLA) are directed against the phospholipid diphosphatidylglycerol (cardiolipin), which is a complex of cardiolipin and the plasma protein β2-glycoprotein-1 (β2-GP1).

Anti-phospholipid syndrome (APS), also called Hughes syndrome, is an autoimmune disease which is characterised by thrombophilia. Cumulative haematological signs are mainly venous (37%) or arterial (27 to 49%) thrombosis, haemocytopenia (30 to 38%), pregnancy complications (55 to 74%), neurological failures (66%) and cardiological (27%), pulmonary (20 to 30%) or cutaneous (40%) tissue damage due to the above-mentioned circulatory disorders. Other APS-induced organ manifestations can include Addison's disease caused by thrombosis of the suprarenal vessels, intestinal necrosis caused by occlusion of the intestinal vessels, Budd-Chiari syndrome caused by hepatic venous thrombosis, and liver and spleen infarction.

APS is an immunocoagulopathy and the most frequently acquired hypercoagulability. 82% of APS patients are women and 18% men. Around 10% of APS cases are familial.

APS is divided into primary APS (pAPS) and secondary APS (sAPS). These are characterised by the same haematological immune responses. In sAPS, however, they occur during the course of the disease as secondary reactions, most frequently in connection with rheumatic diseases (e.g. SLE). Around 1% of APS patients suffer from the most extreme form of APS, the so-called catastrophic form (cAPS), which is characterised by life-threatening multi-organ failure.

APS is proven when at least one clinical APS criterion (thrombosis or pregnancy complications) and one laboratory criterion (medium to high serum/plasma antibody titers measured at an interval of at least 12 weeks) are fulfilled. Antibody detection encompasses lupus anticoagulans (LA), anti-cardiolipin antibodies of class IgG and/or IgM (titer > 99th percentile, measured using ELISA) or anti-β2-glycoprotein-1 antibodies of class IgG and/or IgM (titer > 99th percentile, measured using ELISA).

According to the "International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS) 2006" the **presence of anti-cardiolipin antibodies** is a serological criterion in APS diagnostics. ACA have a high prevalence of 60 to 90% (ACA-IgG 44%, ACA-IgM 12%, ACA IgG/IgM 88%) and persist for more than 12 weeks in APS patients. ACA can be found in the serum of 15 to 30% of sAPS patients. In persons with thrombosis in their anamnesis the prevalence is 20% to 30%.

The specificity of ACA is slightly limited since ACA can also be detected in infections. They occur temporarily in for example syphilis, borreliosis or malaria without the cofactor β2-GP1, which means that they are not associated with APS. A positive ACA result should always be assessed after 12 weeks to confirm APS diagnosis.

Persisting high ACA titers are considered as a risk factor for thrombosis and vascular complications such as cardiac or cerebral infarction, which develop with a probability of 80%.

The serological detection rate in APS diagnostics can be increased to almost 100% by parallel investigation of ACA and β2-GP1 antibodies. In suspected cases of APS that are negative for IgG and IgM isotypes of ACA and anti-β2-GP1, the IgA isotope should also be determined for both autoantibodies.

Antigen	Disease	AAb prevalence
Cardiolipin	Primary anti-phospholipid syndrome (pAPS) Secondary anti-phospholipid syndrome (sAPS)	60% - 90% 20% - 40%

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