Anti-β2-Glycoprotein 1 ELISA (IgM)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG-CLASS	SUBSTRATE	FORMAT
EA 1632-9601 M	β2-glycoprotein 1	IgM	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 IgM class antibodies against β2-glycoprotein 1 (β2-GP1)in serum or plasma. The test kit contains microtiter strips each with 8 break-off reagent wells coated with β2-GP1. In the first reaction step, diluted patient samples are incubated with the wells. In the case of positive samples, specific IgM antibodies (also IgA and IgG) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgM (enzyme conjugate) catalysing a colour reaction.

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 1 200 RU/ml (IgM, human), ready for use	dark red	1 x 2.0 ml	CAL 1
3. Calibrator 2 20 RU/ml (IgM, human), ready for use	red	1 x 2.0 ml	CAL 2
4. Calibrator 3 2 RU/ml (IgM, human), ready for use	light red	1 x 2.0 ml	CAL 3
5. Positive control (IgM, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
6. Negative control (IgM, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
7. Enzyme conjugate peroxidase-labelled anti-human IgM (goat), ready for use	red	1 x 12 ml	CONJUGATE
8. Sample buffer ready for use	light blue	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. Protocol with reference values	---	1 protocol	

LOT Lot — CE — Storage temperature

IVD In-vitro-determination — Unopened usable until

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. Other reagents do not need to be collected separately, unless stated otherwise in official regulations.

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:** Ready for use. The enzyme conjugate must be mixed thoroughly before use.

- **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 ready-to-use diluted 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: The control sera 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.

Preparation and stability of the patient samples

Sample material: 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.

(Partly) manual test performance

Sample incubation: (1. 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).

Wash:
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 of 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.

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

Wash:
Empty the wells. Wash as described above.

Substrate incubation: (3. 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 the reaction:
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.

Test performance using fully automated analysis devices

Sample dilution and test performance are carried out fully automatically using the 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 and this EUROIMMUN ELISA. Validation documents are available on inquiry.
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 1-4 is an example for the **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

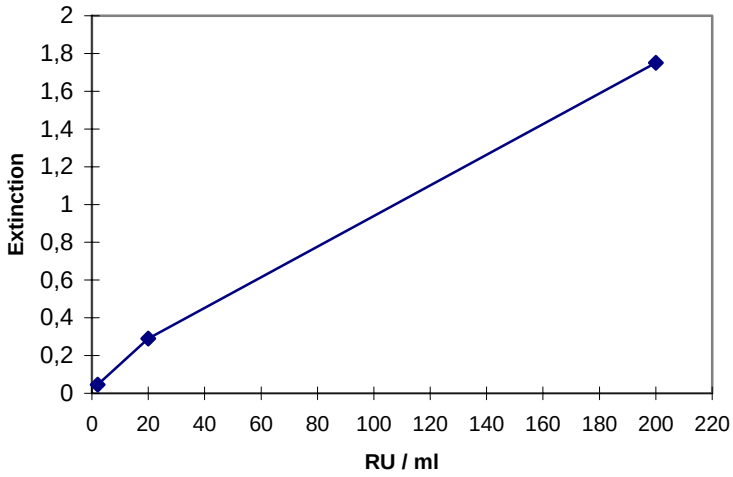
Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of the calibrator 2. Calculate the ratio according to the following formula:

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

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 of a serum sample lies above the value of calibrator 1 (200 RU/ml). The result shold be given as “>200 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 20 relative units (RU)/ml. EUROIMMUN recommends interpreting results as follows:

<20 RU/ml:	**negative**
≥20 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.

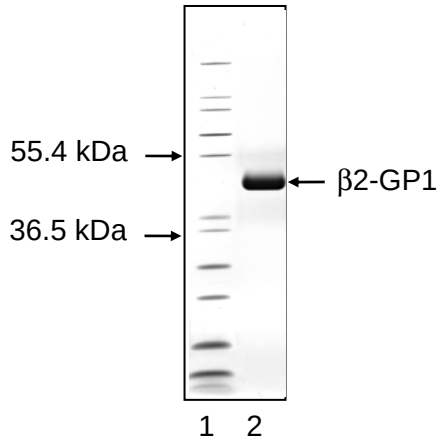
Test characteristics

Calibration: As no international reference serum exists for the measurement of antibodies against β2-glycoprotein 1, 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 or ratios determined for the positive and negative controls must lie within the limits stated for the relevant test kit lot. A protocol 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 highly purified β2-glycoprotein 1 isolated from human serum. The purity of the preparation was verified by SDS polyacrylamid gelelectrophoresis (4 % to 12 % gel). The adjacent figure shows a molecular weight marker on track 1 of the electrophoresis, and the purified β2-GP1 applied to track 2. β2-GP1 has a molecular weight of 50 kDa, its function is not known. β2-GP1 is found in human plasma, both in the free state as well as associated with low-density lipoproteins.



Linearity: The linearity of the test was investigated using series dilutions of patient sera with high antibody concentrations. The Anti-β2-Glycoprotein 1 ELISA (IgM) is linear in the measurement range 11 - 182 RU/ml.

Detection limit: The lower detection limit is defined as the mean value of an analyte-free sample plus three times standard deviation and is the smallest detectable antibody titer. The lower detection limit of the Anti-β2-Glycoprotein 1 ELISA (IgM) is 0.8 RU/ml.

Cross reactivity: The present ELISA specifically detects IgM class antibodies directed against β2-Glycoprotein 1. There were no cross reactions with other autoantibodies in anti-dsDNA positive patient samples.

Interference: Haemolytic, lipaemic and icteric samples showed no influence at 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 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	24	10.6
2	60	7.0
3	63	5.7

Inter-assay-variation, n = 4 x 6		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	26	10.5
2	64	8.3
3	73	6.6

Prevalence and specificity: The prevalence anti-β2-GP1 antibodies (IgM) in a panel of 21 APS patients was 52%. The specificity in a control panel of patients with HIV, HBV or HCV (n=168), healthy pregnant women (n= 200) and healthy blood donors (n=206) was 97.5%.

Reference range: The levels of the anti-β2-GP1 antibodies (IgM) were analyzed with this EUROIMMUN ELISA in 206 healthy blood donors. With a cut-off of 20 RU/ml, 1.0% of the blood donors were anti-β2-GP1 positive (IgM).

Clinical significance

Antibodies against phospholipids or phospholipid-protein complexes (aPL) are associated with the clinical picture of antiphospholipid syndrome (APS). APS encompasses a very comprehensive group of diseases of autoimmune genesis. Among the most important clinical manifestations are: venous and arterial thrombosis, thrombocytopenia, habitual abortion, cardiomyopathy, cardiac, cerebral and renal infarction and pulmonary hypertonia. The artificially created term APS is today divided into the two groups of primary and secondary antiphospholipid syndromes. Characteristic for primary APS (PAPS) are thrombotic complications and antibodies against phospholipids or phospholipid-protein complexes. The term secondary APS (SAPS) characterises similar disease processes in which the patient is suffering from a further autoimmune disease in addition (e.g. Lupus erythematosus disseminatus, scleroderma).

Serologically, various antibodies are found in patients with antiphospholipid syndrome. Of particular diagnostic significance today is the determination of antibodies against cardiolipin and β2 glycoprotein 1. β2 glycoprotein 1 (β2-GP1 or apolipoprotein H) was described for the first time as a plasma protein in 1961 and characterised through molecular biology in 1984. This protein functions as a co-factor for the binding of antibodies to the phospholipid cardiolipin.

In the case of APS patients, IgG and/or IgM class antibodies against β2-GP1 occur with a prevalence of 30 % - 60 %, but they can also be found in asymptomatic subjects. Significant correlations can be found retrospectively between the antibody concentrations and a previous venous thrombosis, IgM antibodies correlate well with arterial thrombosis. No significant correlation has been found between APS-associated abortions and β2-GP1 antibody titres.

The determination of antibodies against β2-GP1 in APS patients achieves diagnostic significance through the possibility of improving the prognosis of thromboembolic complications: β2-GP1 antibodies are only found in the case of autoimmune diseases, whereas antibodies against cardiolipin can be detected in APS and in certain infections (syphilis, borreliosis, AIDS, hepatitis, tuberculosis). In the course of extensive testing, β2-GP1 antibodies have taken on the role of a marker for autoimmune thrombosis. Detection of these antibodies provides a serological aid for the differentiation of autoimmune diseases from infections; the determination of antibodies against cardiolipin does not perform this classification.

β2-GP1 antibodies frequently occur in combination with cardiolipin antibodies. The antibody titre correlates well with cardiolipin concentrations, and in various investigations have shown a high specificity of about 98 % for antiphospholipid syndrome; cardiolipin antibodies achieve a specificity of only 75 %. The sensitivity, at 54 %, appears, however, to be lower than for antibodies against cardiolipin. In addition, the seriousness of a thrombosis in the case of Lupus erythematosus disseminatus correlates well with the titre of antibodies against β2-GP1.

Literature references

1. Amengual O, Atsumi T, Khamashta MA, et al. **Specificity of ELISA for antibody to beta 2-glycoprotein I in patients with antiphospholipid syndrome.** Br J Rheumatol 35: 1239-1243 (1996).
2. Horbach DA, van-Oort E, Donders RC, et al. **Lupus anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with systemic lupus erythematosus. Comparison between different assays for the detection of antiphospholipid antibodies.** Thromb Haemost 76: 916-924 (1996).
3. McNeil HP, Simpson RJ, Chesterman CN, et al. **Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2-glycoprotein I (apolipoprotein H).** Proc Natl Acad Sci USA 87: 4120-4124 (1990).

4. Navarro M, Cervera R, Teixido M, et al. **Antibodies to endothelial cells and beta 2-glycoprotein I in the antiphospholipid syndrome: prevalence and isotype distribution.** Br J Rheumatol 35: 523-528 (1996).
5. Pengo V, Biasiolo A, Brocco T, et al. **Autoantibodies to phospholipid-binding plasma proteins in patients with thrombosis and phospholipid-reactive antibodies.** Thromb Haemost 75(5): 721-724 (1996).
6. Sanmarco M, Soler C, Christide C, et al. **Prevalence and clinical significance of IgG isotype anti-beta 2-glycoprotein I antibodies in antiphospholipid syndrome: a comparative study with anticardiolipin antibodies.** J Lab Clin Med. 129: 499-506 (1997).
7. Teixido M, Font J, Reverter JC, et al. Anti-beta 2-glycoprotein I antibodies: a useful marker for the antiphospholipid syndrome. Br J Rheumatol 36: 113-116 (1997).