研究用試薬

Anti-PLA2R ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EA 1254-9601 G	PLA2R	IgG	Ag-coated microplate wells	96 x 01 (96)

Indication: membranous glomerulonephritis (MGN).

Application: Autoantibodies of class IgG against phospholipase A2 receptors are highly specific for the diagnosis of primary MGN and can be detected in the serum of up to 70%-75% of patients. The ELISA allows qualitative and quantitative determination of human autoantibodies of class IgG against PLA2 receptors. Therapy outcome can be monitored by measuring the anti-PLA2R antibody titer. A titer increase, decrease or disappearance generally precedes a change in the clinical status. Thus, the determination of the antibody titer has a high predictive value with respect to clinical remission, relapse or risk assessment after kidney transplantation.

Principles of the test: The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human autoantibodies of the IgG class against PLA2R in serum or plasma. The test kit contains microtiter strips each with 8 break-off reagent wells coated with PLA2R. In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, 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.

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 2 RU/ml (IgG, human), ready for use	red	1 x 1.0 ml	CAL 1
3. Calibrator 2 20 RU/ml (IgG, human), ready for use	red	1 x 2.0 ml	CAL 2
4. Calibrator 3 100 RU/ml (IgG, human), ready for use	red	1 x 1.0 ml	CAL 3
5. Calibrator 4 500 RU/ml (IgG, human), ready for use	red	1 x 1.0 ml	CAL 4
6. Calibrator 5 1500 RU/ml (IgG, human), ready for use	red	1 x 1.0 ml	CAL 5
7. Positive Control (IgG, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
8. Negative control (IgG, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
9. Enzyme conjugate peroxidase-labelled anti-human IgG (rabbit), ready for use	green	1 x 12 ml	CONJUGATE
10. Sample buffer ready for use	light blue	1 x 100 ml	SAMPLE BUFFER
11. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
12. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
13. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
14. Test instruction	---	1 booklet	
15. Quality control certificate	---	1 protocol	

LOT	Lot description		Storage temperature
IVD	In vitro diagnostics	CE	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:** 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 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, control and incubated microplate strips shoud be handled as infectious waste. All reagents should be disposed of according to official regulations.

Warning: 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

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 **1:101** in sample buffer. For example: dilute 10 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 **qualitative/semiquantative analysis** incubate **calibrator 2** along with the positive and negative controls and patient samples. For **quantitative analysis** incubate **calibrators 1 to 5** along with the positive and negative controls and patient samples.

Partly) manual test performance

Sample incubation: (1st step)
Transfer 100 µl of the positive control, negative control 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 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.

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 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 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 the Analyzer I-2P 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 2	P 10	P 18		
B	pos.	P 7	P 15	P 23			C 2	P 3	P 11	P 19		
C	neg.	P 8	P 16	P 24			C 3	P 4	P 12	P 20		
D	P 1	P 9	P 17	P 25			C 4	P 5	P 13	P 21		
E	P 2	P 10	P 18				C 5	P 6	P 14	P 22		
F	P 3	P 11	P 19				pos.	P 7	P 15	P 23		
G	P 4	P 12	P 20				neg.	P 8	P 16	P 24		
H	P 5	P 13	P 21				P 1	P 9	P 17	P 25		

The pipetting protocol for microtiter strips 1-4 is an example for the **qualitative/semiquantitative analysis** of 25 patient sera (P 1 to P 25).
The pipetting protocol for microtiter strips 7-10 is an example for the **quantitative analysis** of 25 patient sera (P 1 to P 25).

The calibrators (C 1 to C 5), 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 further improved by duplicate determinations for each sample. 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 <0,7:	**negative**
Ratio ≥0,7 to <1,0:	**borderline**
Ratio ≥1,0:	**positive**

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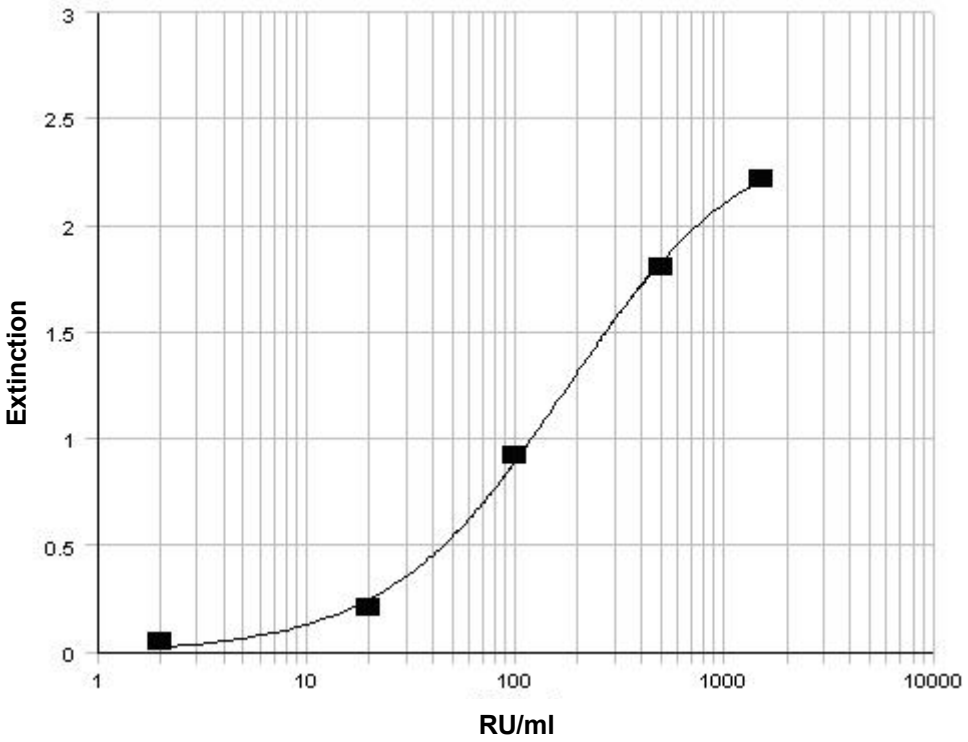
Quantitative: The standard curve from which the antibody concentration in the unknown sample can be taken is obtained by plotting the extinction values measured for the 5 calibration sera (linear, y-axis) against the corresponding concentrations (logarithmic, x-axis). The standard curve can be calculated by computer using one of the following curve-fitting techniques: 4-parameter logistic, 5-parameter logistic, spline fits, log/logit curve and lim/limit curve. 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 that of calibrator 5 (1500 RU/ml), the result should be reported as “>1500 RU/ml”. It is recommended that the sample be remeasured in a new test run at a dilution of e.g. 1:400. 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:

<14 RU/ml:	**negative**
≥14 to <20 RU/ml:	**borderline**
≥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 antibodies against PLA2R antigen, the calibration is performed in relative units (RU).

For every group of tests performed, the extinction values of the calibrators and the relative units and/or ratios 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 reagent wells are coated with recombinant phospholipase A2 receptors. The recombinant protein based on human cDNA was produced in a human cell line.

Linearity: The linearity of the Anti-PLA2R ELISA (IgG) was determined by assaying at least four serial dilutions of different patient samples. The coefficient of determination R^2 for all sera was > 0.95. The Anti-PLA2R ELISA (IgG) is linear at least in the tested concentration range (6 RU/ml to 1500 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-PLA2R ELISA (IgG) is 0.6 RU/ml.

Cross reactivity: The ELISA specifically detects antibodies of class IgG directed against PLA2R. There were no cross reactions with other autoantibodies in samples of patients with thyroiditis (n = 5), Sjögren's syndrome (n = 5), SSc (n = 5) and RA (n = 5).

Interference: Haemolytic, lipaemic and icteric samples showed no influence on 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 using three sera. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on three determinations performed in 10 different test runs.

Intra-assay variation, n = 20		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	26	3.4
2	97	1.7
3	861	5.7

Inter-assay variation, n = 3 x 10		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	28	4.2
2	99	6.4
3	878	10.3

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Clinical sensitivity and specificity: Sera from 122 patients with primary membranous glomerulonephritis (pMGN), from a control panel of 342 patients with other diseases and 191 healthy blood donors were investigated using the EUROIMMUN Anti-PLA2R ELISA. The sensitivity of the ELISA for pMGN was 97.5%, at a specificity of 100%, including borderline results.

n = 122		IIFT	
		positive	negative
ELISA	positive	118	0
	borderline	1	0
	negative	3	0

Panel	**Anti-PLA2R ELISA**	
	n	**(specificity) borderline / positive**
Other glomerulonephritides	181	0 (100%)
Other autoimmune diseases	161	0 (100%)
Blood donors	191	0 (100%)
Total	**533**	**0 (100%)**

Reference range: Levels of anti-PLA2R antibodies were analysed in 191 sera from healthy blood donors using the EUROIMMUN ELISA. The mean concentration of antibodies against PLA2R was 0.4 RU/ml and ranged from 0.0 to 5.0 RU/ml. With a cut-off value of 20 RU/ml, 0% of blood donors were anti-PLA2R positive.

Cut-off	**Percentile**
3.8 RU/ml	99.
5.0 RU/ml	100.

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Clinical Significance

The detection of autoantibodies against phospholipase A2 receptors (PLA2R) is useful in the diagnosis of primary membranous glomerulonephritis (MGN, pMGN), which has formerly been known as idiopathic membranous nephropathy (IMN). MGN is a chronic inflammatory disease of the renal corpuscle (glomeruli), which is accompanied by a progressive reduction in kidney function.

The autoimmune mechanism of MGN, which was first discovered and described in 2009, is the result of autoantibodies reacting with PLA2R (transmembrane glycoproteins), which are expressed in human glomeruli on the surface of podocytes. They are involved in regulatory processes in the cell following phospholipase binding. Up until now two main groups of PLA2R have been described (types M and N), with the M type of the PLA2 receptor being identified as the major target antigen of autoantibodies. Autoantibodies against PLA2R are suspected to be pathogenetically relevant, although the exact pathogenesis is still unknown. Circulating autoantibodies expressed by MGN patients bind to PLA2R on podocytes. Immune complexes are produced in situ in the area of the glomerular basement membrane. There, they might trigger complement activation, which causes damage to podocytes and the blood-urine barrier. This leads to protein entering the primary urine, and subsequently proteinuria. Alternatively, the autoantibodies can also act as receptor agonists or antagonists and damage the podocyte architecture and, hence, the barrier function.

MGN is the most frequent kidney disorder with nephrotic syndrome. With increasing proteinuria, the long-term risk of kidney failure with major morbidity and mortality becomes higher, particularly in connection with thromboembolic and cardiovascular complications.

MGN is prevalent in all ethnic groups and genders, with men over 40 years of age and of white skin colour being more frequently affected. In young women with suspected MGN, lupus nephritis should be considered. MGN is rare in children (only 2-3% of kidney disorders in children).

Symptoms in MGN:
- Around 80% of MGN patients suffer from nephrotic syndrome with sometimes severe oedema in the legs and eye lids, weight gain and reduced urination.
- Around 20% of patients have proteinuria without any additional symptoms.
- Around 50% of patients have microscopic haematuria, albuminuria and glucosuria.
- Around 70% of patients show normal blood pressure and kidney function at the onset of the disease.

MGN can proceed in many different ways. In around one third of cases the disease heals spontaneously. In a further third it is stagnant and in the final third it progresses to chronic kidney failure. Acute kidney failure is rare. In chronic cases, complete kidney failure (terminal kidney insufficiency) occurs after five years in approx. 15% of untreated patients, after ten years in approx. 35% and after 15 years in approx. 40%.

Primary membranous glomerulonephritis (pMGN) should be discriminated from secondary membranous glomerulonephritis (sMGN), which is a secondary (accompanying) disease in infections, in drug therapy or abuse or intake of toxins, in collagenosis and other autoimmune diseases and in tumours. sMGN improves with treatment of the underlying disease. The treatment of MGN as an independent disease improves prognosis, particularly with respect to nephrotic syndrome and hypertonicity.

Diagnosis of MGN is made by kidney puncture, histological examination and electron microscopy of the kidney tissue. The deposition of immune complexes on the outside of the glomerular basement membrane is characteristic for the disease.

Serological diagnosis of MGN, however, is less time-consuming and less stressful for the patient. The identification and characterisation of PLA2R (type M) as the target antigen of circulating antibodies in MGN has proven to be of major importance for non-invasive diagnostics. Autoantibodies of class IgG against PLA2R are highly specific for the diagnosis of primary MGN. They can be detected in the serum of up to 70% to 75% patients. They are not exhibited by healthy blood donors or patients with lupus or IgA nephritis.

RC-IFT and ELISA are available for the determination of autoantibodies against PLA2R. The Anti- PLA2R RC-IFT uses transfected cells as standard substrate. The Anti- PLA2R ELISA is based on purified human recombinant receptor from transfected cells. RC-IFT and ELISA are suited for qualitative and quantitative detection of human autoantibodies of class IgG against PLA2R. The success of therapeutic measures can be assessed by means of the anti- PLA2R titer. A titer increase, decrease or disappearance generally precedes a change in the clinical status. Thus, the determination of the autoantibody titer has a high predictive value with respect to clinical remission or relapse and estimation of the risk of relapse after kidney transplantation.

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