Anti-Myeloperoxidase ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG-CLASS	SUBSTRATE	FORMAT
EA 1211-9601 G	Myeloperoxidase (MPO)	IgG	Ag-coated microplate wells	96 x 01 (96)

Indications: Microscopic arteritis, polyarteritis nodosa, Churg-Strauss syndrome.

Principles of the test: The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human autoantibodies of the IgG class against myeloperoxidase (MPO) in serum or plasma. The test kit contains microtiter strips each with 8 break-off reagent wells coated with MPO. 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 1 200 RU/ml (IgG, human), ready for use	dark red	1 x 2.0 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 2 RU/ml (IgG, 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), ready for use	green	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. 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. 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.

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.

Warning: Calibrators 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.

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 **1:101** 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 **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).

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 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 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: (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, Analyzer I-2P or the DSX from Dynex 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), 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

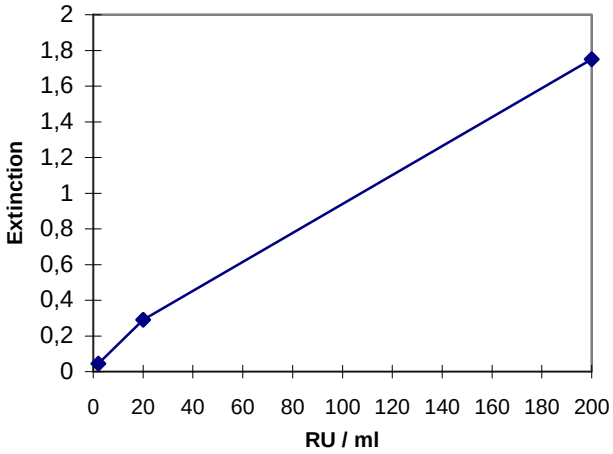
Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of calibrator 2. Calculate the ratio according to the following formula:

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

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 patient sample lies above the value of calibrator 1 (200 RU/ml, the result should be given as ">200 RU/ml". It is recommended that the sample be re-tested at a dilution of 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:

<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 antibodies against MPO, 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 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 microplate wells were coated with highly purified myeloperoxidase isolated from human granulocytes.

The cytoplasmic granules of the granulocytes contain numerous proteins, among them lactoferrin, myeloperoxidase, elastase, cathepsin G and lysozyme. **Myeloperoxidase** (molecular weight 118 kDa) is involved in the production of oxygen radicals (O_2^-, H_2O_2, OCl^-) which are toxic for many bacteria.

Linearity: The linearity of the Anti-MPO-ELISA (IgG) 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-Myeloperoxidase ELISA (IgG) is linear at least in the tested concentration range (9 RU/ml to 196 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-Myeloperoxidase ELISA (IgG) is 1.5 RU/ml.

Cross reactivity: This ELISA showed no cross reactivity.

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	58	4.4
2	67	2.3
3	125	2.1

Inter-assay variation, n = 4 x 6		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	59	5.1
2	71	4.6
3	125	3.5

Prevalence and specificity: The prevalence in clinically characterized patients with microscopic polyangitis (n = 30) was 53%. The specificity in a panel of control sera (n = 283) from patients with Wegener's granulomatosis and healthy blood donors was 99.6%.

Patient group (n = 313)	Origin of sera	n	Anti-MPO positive (IgG)
Microscopic polyangitis	Prof. Groß, Rheumaklinik Bad Bramstedt	30	16 (53%)
Wegener's granulomatosis	Prof. Groß, Rheumaklinik Bad Bramstedt	77	1 (1%)
Blood donors	Universitätsklinikum Lübeck	206	0

Reference range: Levels of anti-myeloperoxidase antibodies (IgG) were analyzed with this EUROIMMUN ELISA in a panel of 206 healthy blood donors. With a cut-off of 20 RU/ml, all blood donors were anti-Myeloperoxidase negative.

Correlation of the ELISA with the indirect immunofluorescence test (IIFT): 30 sera from patients with microscopic polyangitis (MPA), 327 sera from patients with other autoimmune diseases and 206 sera from healthy blood donors were investigated with the EUROIMMUN Anti-Myeloperoxidase ELISA (IgG) and the EUROIMMUN ANCA IIFT (IgG) was used as reference. The ELISA showed a sensitivity of 93.3% and a specificity of 99.8% with regard to the IIFT.

MPA, n = 30		IIFT (pANCA, formalin-resistent)	
		positive	negative
ELISA	positive	14	2
	negative	1	13

Control group, n = 533		IIFT (pANCA, formalin-resistent)	
		positive	negative
ELISA	positive	2	1
	negative	3	527

Antibodies against granulocyte cytoplasmic antigens

Serological testing for neutrophil granulocyte cytoplasmic antibodies (ANCA) is an important aid in the diagnosis of autoimmune diseases like Wegener's granulomatosis, rapid progressive glomerulonephritis, polyarteritis, ulcerative colitis, primary sclerosing cholangitis. Several methods are used for the detection of ANCA. Standard technique is the indirect immunofluorescence test on ethanol-fixed neutronphil granulocytes. At least two different staining patterns can be differentiated: a granular fluorescence in the cytoplasm of the granulocytes (cANCA: cytoplasmic pattern, Wegener's granulomatosis) and a smooth or fine granular fluorescence around the cell nuclei of the granulocytes (pANCA: perinuclear pattern). The cANCA pattern is created by antibodies to proteinase 3. As target antigens of pANCA lactoferrin, myeloperoxidase, elastase, cathepsin G, lysozyme and ß-glucuronidase has been identified up to now.

The indirect immunofluorescence is a screening test for all autoantibodies to granulocytes. But, the corresponding antigens of pANCA cannot be differentiated by the immunofluorescence test. For a differentiation of the target antigens of pANCA purified and characterized proteins are used as substrates (EUROIMMUN ANCA Profile ELISA or monospecific ELISA). Occasionally, pANCA are detected in the immunofluorescence test which do not react with one of the above mentioned antigens: Obviously not all of the relevant antigens are known.

Clinical significance

pANCA induced by antibodies against MPO are mainly associated with microscopic arteritis. Moreover, MPO-ANCA occur in classic polyarteritis nodosa, Churg-Strauss syndrome and Goodpasture syndrome. In rare cases they are found in lupus erythematosus disseminatus and rheumatoid arthritis. Antibodies against MPO of the IgA class are described in patients with Henoch-Schönlein purpura.

Autoantibodies to granulocytes are found in a number of diseases:

Associated Diseases	**Fluorescence pattern**	**Antigens**
Wegener's granulomatosis	cANCA, rarely pANCA	PR3, rarely MPO
Microscopic arteritis	cANCA, pANCA	PR3, MPO
Churg-Strauss-Syndrome	pANCA	MPO
Polyarteritis nodosa	ANCA (low percentage)	rarely PR3 oder MPO
Rheumatoid arthritis	pANCA, atypical ANCA	rarely MPO, Lactoferrin
Disseminated lupus erythematodes	pANCA	rarely MPO, Lactoferrin
Ulcerative colitis (57%) Primary sclerosing cholangitis Crohn's disease (7%)	pANCA, atypical ANCA	Cathepsin G, Lactoferrin, Elastase, Lysozyme other unknown antigens
Autoimmune hepatitis	pANCA, atypical ANCA	

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