Anti-PR3-hn-hr ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EA 1201-9601-2 G	Proteinase 3 (PR3), recombinant + native	IgG	Ag-coated microplate wells	96 x 01 (96)

Indication: The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human autoantibodies of the immunoglobulin class IgG against proteinase 3 (PR3) in serum or plasma for the diagnosis of granulomatosis with polyangiitis (Wegener's) (GPA).

Application: For optimal ANCA diagnostics, the International Consensus Statement recommends the parallel use of an indirect immunofluorescence test and Anti-PR3 ELISA or Anti-MPO ELISA. The combination of both test systems provides the highest specificity and sensitivity for the diagnosis of small-vessel vasculitis.
The reagent wells of the Anti-PR3-hn-hr ELISA are coated with a mixture of recombinant PR3 and native PR3. Thus, the test has a very good specificity (99 %) with a significantly higher sensitivity (94 %) than those ELISAs using only native antigens. The superior sensitivity of the Anti-PR3-hn-hr ELISA and its suitability for detecting relapses in patients under treatment has been described in Damoiseaux et al. (Annals of the Rheumatic Diseases).

Principles of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with a mixture of recombinant human and native human PR3. 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 diagnostic medical device			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 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 must be incubated within one working day.

Sample dilution: Patient samples are diluted to be investigated are diluted **1:101** with sample buffer.
Example: Add 10 µl of sample to 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 **qualitative/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 and the Analyzer I-2P 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 **qualitative/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

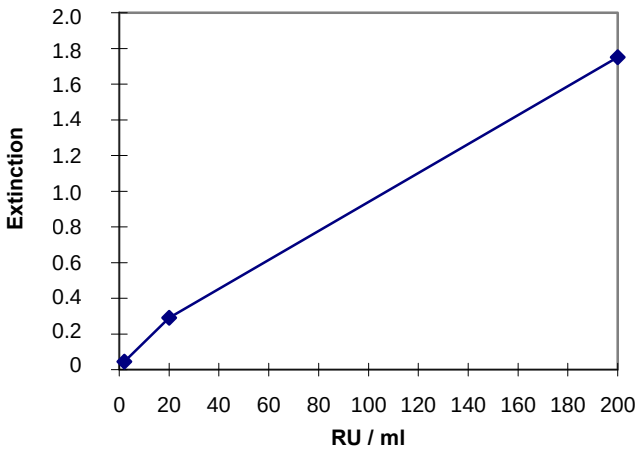
Qualitative/semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of calibrator 2. Calculate the ratio according to the following formula:

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

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 for a patient sample lies above the value of calibrator 1 (200 RU/ml), the result should be reported as "> 200 RU/ml". It is recommended that the sample be retested 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:

<20 RU/ml:	**negative**
≥20 RU/ml:	**positive**

The recommendation is based on data yielded in a ROC analysis using the results of 140 ANCA-associated vasculitis patients (cANCA positive) and 1014 control samples. The specificity achieved was 99%, at a cut-off of 17.6 RU/ml. The 99th percentile with respect to 429 healthy blood donors was 17.7 RU/ml (see respective paragraphs in "Test characteristics").

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: As no international reference serum exists for antibodies against PR3, the calibration is performed in relative units (RU).

For every group of tests performed, the relative units or ratio values 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: The microplate wells were coated with a mixture of recombinant and native proteinase 3. The recombinant protein is based on human cDNA and was produced in a human cell line. The native proteinase 3 was isolated from human neutrophils.

Linearity: The linearity of the Anti-PR3-hn-hr ELISA (IgG) was determined by assaying at least 4 serial dilutions of different patient samples. The Anti-PR3-hn-hr ELISA (IgG) is linear at least in the tested concentration range (4 RU/ml to 197 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-PR3-hn-hr ELISA (IgG) is 0.6 RU/ml.

Cross reactivity: This ELISA specifically detects autoantibodies of class IgG against PR3. When investigating patient sera for autoantibodies against lactoferrin (n = 1), elastase (n = 4) and MPO (n = 7), and samples from patients with the diseases ulcerative colitis (n = 10), Crohn's disease (n = 10) and primary bilary cirrhosis (n = 15) no cross reactions were found.

Interference: Haemolytic, lipaemic and icteric samples showed no influence on the result up to a concentration of 10.0 mg/ml for haemoglobin, 20.0 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 7 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 (RU/ml)**	**CV (%)**
1	11	5.2
2	19	2.6
3	21	2.8
4	55	4.1
5	89	2.6
6	108	1.8
7	152	2.8

Inter-assay variation, n = 4 x 6		
Sample	**Mean value (RU/ml)**	**CV (%)**
1	12	6.2
2	19	5.4
3	20	5.9
4	47	11.2
5	85	4.3
6	106	4.2
7	159	3.9

Clinical sensitivity and specificity: Sera from 163 ANCA-associated vasculitides (AAV) patients (cANCA positive), a control panel of 585 patients with other diseases and 429 healthy blood donors were analysed using the EUROIMMUN Anti-PR3-hn-hr ELISA (IgG). The sensitivity of the ELISA for cANCA positive AAV patients was 94%, with a specificity of 99%.

Panel		**Anti-PR3-hn-hr ELISA**	
		n	**positive**
AAV (cANCA positive)	**Biopsy-proven AAV**	**58**	**55 (94.8%)**
	AAV outpatients	**35**	**33 (94.3%)**
	AAV, relapses	**23**	**23 (100%)**
	Granulomatosis with polyangiitis (Wegener's) (GPA)	**47**	**43 (91.5%)**

Sensitivity with respect to IIFT (cANCA)	**163**	**154 (94.5 %)**
Non-ANCA-associated vasculitis (e.g. cryoglobulinemia, Henoch-Schonlein purpura, large vessel vasculitides)	**55**	**0**
Rheumatoid arthritis	**230**	**0**
Systemic lupus erythematosus	**100**	**0**
Sjogren's syndrome	**200**	**1 (0.5%)**
Asymptomatic blood donors	**429**	**4 (0.9%)**
Specificity	**1014**	**5 (99.5%)**

In a ROC analysis of the results (AUC: 0.984) of 140 ANCA-associated vasculitis patients (cANCA positive) and 1014 control samples listed in the above table the following characteristics were determined:

cut-off	specificity	sensitivity
4.9 RU/ml	95%	96%
12.0 RU/ml	98%	95%
17.6 RU/ml	99%	94%

Reference range: Levels of anti-PR3 were analysed in 429 sera from healthy blood donors between 19 and 69 years of age (172 women, 257 men) using the EUROIMMUN ELISA. No differences with respect to age or gender were observed. The mean concentration of antibodies against PR3 was 2.2 RU/ml (± 9.9 RU/ml of standard deviation) and the values ranged from 0.1 to 171.7 RU/ml. With a cut-off of 20 RU/ml 4 blood donors were anti-PR3 positive.

cut-off	percentile
4.4 RU/ml	95.0%
12.5 RU/ml	98.0%
17.7 RU/ml	99.0%

Clinical significance

ANCA are autoantibodies directed against antigens found in cytoplasmic granules of neutrophils and monocytes [1, 2]. ANCA are classified according to the immunfluorescence patterns they produce on normal neutrophils [3, 4] and according to their target antigens. ANCA can be determined by using various methods [5]. Indirect immunofluorescence (IIF) containing ethanol-fixed neutrophil granulocytes as substrate is considered as the standard method. At least two fluorescence patterns can be differentiated: a granular fluorescence which is distributed regularly over the entire cytoplasm of the granulocytes, leaving the cell nuclei free (cANCA: cytoplasmic pattern), and a predominantly smooth, partly fine granular fluorescence wrapped ribbon-like around the cell nuclei of the granulocytes (pANCA: perinuclear pattern).

ANCA are typically found in granulomatosis with polyangiitis (Wegener's) (GPA), microscopic polyangiitis (MPA) including renal limited vasculitis, and Churg-Strauss syndrome (CSS), which are all forms of small-vessel vasculitis [2, 3, 6, 7]. These three diseases are grouped together as ANCA-associated vasculitides (AAV) according to the widely accepted classification system introduced by the Chapel Hill Consensus Conference [9]. Classical cANCA are present in most patients with GPA (more than 90% in generalised GPA with glomerulonephritis, 70% in limited GPA without glomerular involvement), and in about 30% of patients with MPA. Classical cANCA is almost always directed against proteinase 3 (PR3), and very rarely against myeloperoxidase (MPO) or against both PR3 and MPO simultaneously [5, 8, 10, 11, 12, 13, 14]. Some cANCA exhibit a flat homogeneous cytoplasmic staining in IIF (mostly termed atypical cANCA) which is often directed against bactericidal/permeability increasing protein (BPI) [9]. ANCA have also been reported in rare cases of many different infections, including suppurative lung

disease, Pseudomonas infections in cystic fibrosis, and in subacute bacterial endocarditis. With infections, the IIF pattern is often atypical cANCA and the antigens commonly include BPI [11].

In patients with MPA and CSS, MPO is the main target antigen of pANCA [4, 16]. pANCA could also be found in patients with a wide range of non-vasculitic disorders such as inflammatory bowel diseases [17, 18], primary sclerosing cholangitis, autoimmune liver diseases, collagenosis, rheumatoid arthritis, malignancies and infections. In these disorders, ANCA are almost always directed against neutrophil constituents other than MPO. In addition to MPO other proteins have been identified as target antigens of pANCA: lactoferrin, elastase, BPI, cathepsin G, lysozyme and ß-glucuronidase. Occasionally, pANCA are found in immunofluorescence which do not react with one of the above antigens: apparently, not all of the relevant antigen-antibody systems have yet been found [11, 19, 20].

Current criteria for the diagnosis of GPA, MPA and CSS rely on histology and do not take ANCA status or antibody specificity into account [8, 9, 21]. However, histology is not always conclusive, and clinicians are using ANCA to help confirm or exclude the diagnosis of small-vessel vasculitis, and to monitor inflammatory activity in these diseases. In other disease conditions such as inflammatory bowel diseases, primary sclerosing cholangitis, autoimmune liver diseases, Felty's syndrome, scleroderma, and SLE, ANCA determination has been suggested to help in differential diagnosis or to provide some additional information [19].

With Indirect immunofluorescence, all autoantibodies against granulocytes can be detected. The International Consensus Statement has recommended screening for ANCA by IIF and confirmation of IIF-positive sera in both Anti-PR3 and Anti-MPO ELISA [2]. The use of IIF or ELISA results alone had unsatisfactory diagnostic specificity with respect to disease control sera. In contrast, the combination of IIF with Anti-PR3 ELISA and Anti-MPO ELISA showed 99% specificity for the diagnosis of small vessel vasculitis. Using this approach, the sensitivity for newly diagnosed GPA and MPA was 73% and 67%, respectively [22, 23].

The most important clinical symptoms of ANCA-associated vasculitides are caused by poor blood supply to organs or formation of aneurysms and bleeding due to destruction of blood vessels. GPA is a febrile, chronic granulomatosis disease, mainly of the nasopharynx, lungs and kidney. Since cANCA have been investigated, the number of diagnoses of GPA has tripled. Based on the high specificity of cANCA the number of diagnosed early-stage and abortive cases of GPA increases steadily [24, 25].

While in most ANCA-associated vasculitis patients a cANCA pattern is due to reactivity with PR3-ANCA, some cANCA positive sera do not react with PR3. Therefore a mixture of human native (hn) and human recombinant (hr) PR3 was developed and used as antigen. The recombinant protein was based on human cDNA and produced in a human cell line. The native PR3 was isolated from human neutrophils. The resulting Anti-PR3-hn-hr ELISA (IgG) assay has an excellent diagnostic performance with very high sensitivity. This feature is combined with a good predictability of clinical relapses in PR3-ANCA-associated vasculitis patients [8, 14].

The demonstration of ANCA antibodies is helpful in both the diagnosis and management of GPA and MPA. ANCA levels are usually high at presentation, fall with treatment and often increase prior to clinical relapse. A rather good association of ANCA titers with disease activity has been described by many scientific researchers. However, increasing ANCA titers do not reliably predict relapse. The role of ANCA in monitoring disease activity in patients with vasculitis and the significance of changes in ANCA titers for treatment remains unclear [26, 27].

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