# ANCA Profile ELISA (IgG)
# Test instruction

| ORDER NO. | ANTIBODIES AGAINST | IG CLASS | SUBSTRATE | FORMAT |
|---|---|---|---|---|
| EA 1200-1208-1 G | separate: proteinase 3, lactoferrin, myeloperoxidase, elastase, cathepsin G, BPI | IgG | Ag-coated microplate wells | 12 x 08 (96) |

**Indications:** The ELISA test kit provides a semiquantitative in vitro assay for human autoantibodies of the immunoglobulin class IgG against six different antigens **(proteinase 3, lactoferrin, myeloperoxidase, neutrophil elastase, cathepsin G and BPI (bactericidal/permeability increasing protein))** in serum or plasma for the diagnosis of Wegener's granulomatosis, Microscopic arteritis, Churg-Strauss syndrome, Polyarteritis nodosa, Colitis ulcerosa, Crohn's disease and Autoimmune hepatitis.

**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 ANCA Profile ELISA, moreover, allows exact differentiation of further ANCA specificities, e.g. elastase, cathepsin G, BPI or lactoferrin, which can be found in chronic-inflammatory bowel diseases.

**Principle of the test:** The test kit contains microtiter strips each with 8 reagent wells separately coated with these six antigens. In the first reaction step, diluted patient samples are incubated with 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**<br>12 microplate strips each containing 8 wells in a frame, ready for use:<br>1. no antigen, 2. mixed antigen, 3. proteinase 3, 4. lactoferrin, 5. myeloperoxidase; 6. neutrophil elastase, 7. Cathepsin G, 8. BPI | --- | 12 x 8 | STRIPS |
| **2. Calibrator**<br>(human, IgG), ready for use | dark red | 1 x 2.0 ml | CAL |
| **3. Enzyme conjugate**<br>peroxidase-labelled anti-human IgG (rabbit), ready for use | green | 1 x 12 ml | CONJUGATE |
| **4. Sample buffer**<br>ready for use | light blue | 1 x 100 ml | SAMPLE BUFFER |
| **5. Wash buffer**<br>10x concentrate | colourless | 1 x 100 ml | WASH BUFFER 10x |
| **6. Chromogen/substrate solution**<br>TMB/$H_2O_2$, ready for use | colourless | 1 x 12 ml | SUBSTRATE |
| **7. Stop solution**<br>0.5 M sulphuric acid, ready for use | colourless | 1 x 12 ml | STOP SOLUTION |
| **8. Test instruction** | --- | 1 booklet | |
| **9. 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.

- **Calibrator:** Ready for use. The calibrator 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 calibrator of human origin has 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: Patient samples** are diluted **1:101** in sample buffer.
For example: dilute 10 µl sample in 1.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

NOTE: The calibrator is prediluted and ready for use, do not dilute it.

## Incubation

**(Partly) manual test performance**

**Sample incubation:** (1st step)
Transfer 100 µl of the sample buffer (blank), calibrator 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.
Individual reagent wells may not be removed from the holder and are solely to be used batch-specifically, according to the manufacturer's specifications, in this packaging.

**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

Coating
A: no antigen
B: mixed antigen
C: PR3
D: Lactoferrin
E: MPO
F: Elastase
G: Cathepsin G
H: BPI

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Bl | Bl | Bl | Bl | | | | | | | | |
| B | C | C | C | C | | | | | | | | |
| C | P 1 | P 2 | P 3 | P 4 | | | | | | | | |
| D | P 1 | P 2 | p 3 | P 4 | | | | | | | | |
| E | P 1 | P 2 | P 3 | P 4 | | | | | | | | |
| F | P 1 | P 2 | P 3 | P 4 | | | | | | | | |
| G | P 1 | P 2 | P 3 | P 4 | | | | | | | | |
| H | P 1 | P 2 | P 3 | P 4 | | | | | | | | |

The above pipetting protocol is an example of the **semiquantitative analysis** of antibodies in 4 patient samples (P 1 to P 4).
Sample buffer (Bl) and the calibrator (C) have been incubated in the corresponding well of each microplate strip. The patient samples have been incubated in one well for each antigen.
The calibrator (C), sample buffer (Bl) 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.

## Calculation of results

**Semiquantitative:** If the photometer has no automatic blank adjustment, the mean blank value must first be calculated and subtracted from all other measured values. Then the mean extinction value for all measurements of the calibrator is calculated and multiplied by the **factor 0.2**. This provides the upper limit of the normal range (cut-off). Values above the indicated cut-off are to be considered as positive, those below as negative. Besides this qualitative interpretation a semiquantitative evaluation of the result is possible by calculating a ratio according to the following formula:

$$\frac{\textbf{Extinction of the patient sample}}{\textbf{Cut - off extinction}} = \frac{\textbf{Extinction of the patient sample}}{\textbf{Extinction of calibrator x 0.2}} = \textbf{Ratio}$$

**Exception PR3:** For PR3 the calculated ratio is multiplied by the factor 1.4.

$$\frac{\textbf{Extinction of the patient sample}}{\textbf{Cut - off extinction}} = \frac{\textbf{Extinction of the patient sample}}{\textbf{Extinction of calibrator x 0.2}} = \textbf{Ratio x 1.4}$$

EUROIMMUN recommends interpreting results as follows:

| Ratio | | | Finding |
|---|---|---|---|
| <1.0 | | | negative |
| ≥1.0 | to | 2.0 | weak positive |
| ≥2.0 | to | 5.0 | positive |
| ≥5.0 | | | high positive |

An indirect immunofluorescence test should always be performed in parallel to the determination of granulocyte antibodies by ELISA. On the one hand, this provides a check on plausibility as a safeguard against false-positive ELISA results, on the other hand, by using **EUROIMMUN granulocytes**, and in particular **in combination with frozen sections of primate liver**, immunofluorescence permits the detection of a wider range of granulocyte antibodies, as not all granulocyte antigens are presently known.

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 ANCA Profile ELISA is calibrated using a mixed serum. Consequently, the results for each test parameter can show a larger spread than the monospecific EUROIMMUN ELISA: For these ELISA there are specific calibrators and controls for each antigen, and a standard curve consists of several measuring points.

For every group of tests performed, the blank values and the extinction values of the calibrator 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 blank and calibrator 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 calibrator is subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

**Antigens:** The antigens used in this ELISA are all native proteins purified from human neutrophils.

**Proteinase 3** is a neutral serine protease which is capable of degrading extracelullar matrix proteins like elastin, fibronectin and collagen type IV. The enzyme has three isoforms of which the main band has a molecular weight of 26.8 kDa in SDS gel electrophoresis. 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.

**Lactoferrin** (molecular weight 77 kDa) is capable of binding to iron and thus has a bacteriostatic effect because most of the pathogenic bacteria depend on iron for growing.

**Myeloperoxidase** (molecular weight 118 kDa) is involved in the production of oxygen radicals ($O_2^-$, $H_2O_2$, $OCl^-$ ) which are toxic for many bacterias.

**Elastase** (molecular weight 30 kDa) and **cathepsin G** (molecular weight 23.5 kDa) belong to the group of proteases. These enzymes are normally sequestered by binding to the polysaccharide matrix of the granules and are only released during the process of phagocytosis. Both enzymes can degrade connective tissue proteins like elastin, collagens, proteoglycans and fibronectin. Cathepsin G also can cause the release of angiotensin II from angiotensin I.

**BPI** (molecular weight 55 kDa) is a cationic membrane-associated protein which is toxic for Gram-negative bacteria.

**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 ANCA Profile ELISA is ratio 0.01.

**Cross reactivity:** This ELISA showed no cross reactivity.

**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 (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* | |
|---|---|
| **Antigen** | **CV (%)** |
| Proteinase 3 | 2.6 |
| Laktoferrin | 3.5 |
| MPO | 3.8 |
| Elastase | 4.0 |
| Cathepsin G | 11.1 |
| BPI | 3.3 |

| *Inter-assay variation, n = 4 x 6* | |
|---|---|
| **Antigen** | **CV (%)** |
| Proteinase 3 | 4.5 |
| Laktoferrin | 3.5 |
| MPO | 3.8 |
| Elastase | 5.5 |
| Cathepsin G | 11.6 |
| BPI | 4.4 |

**Reference range:** The levels of anti-ANCA antibodies (IgG) were analysed with this EUROIMMUN ELISA in a panel of 200 healthy blood donors. With a cut-off of ratio 1.0 the following prevalences were measured:

| Antibodies against | Prevalence |
|---|---|
| PR3 | 1% |
| Lactoferrin | 0% |
| MPO | 0% |
| Elastase | 0% |
| Cathepsin G | 0% |
| BPI | 1.5% |

The prevalence of the antibody of the class IgG against PR3, lactoferrin, MPO, elastase, cathepsin G and BPI in four different patient collectives:

| Patient panel | n | Anti-PR3 | Anti-LF | Anti-MPO | Anti-EL | Anti-CathG | Anti-BPI |
|---|---|---|---|---|---|---|---|
| **Wegener´s granulomatosis** | **60** | - | 0% | 1.7% | 0% | 0% | 5% |
| **ANCA-associated vasculitis (cANCA positive)** | **163** | 95% | - | - | - | - | - |
| **Microscopic arteritis (MPA)** | **30** | - | 3.3% | 53.3% | 6.7% | 0% | 0% |
| **Churg-Strauss syndrome (CSS)** | **10** | - | 0% | 0% | 0% | 0% | 0% |

## 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 neutrophil 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. Antibodies against BPI can be seen as cANCA as well as pANCA pattern.

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

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 or MPO |
| Rheumatoid arthritis | pANCA, atypical ANCA | rarely MPO, Lactoferrin |
| Disseminated lupus erythematodes | pANCA | rarely MPO, Lactoferrin |
| **Ulcerative colitis** (57%)<br>Primary sclerosing cholangitis<br>Crohn's disease (7%) | pANCA, atypical ANCA | Cathepsin G, Lactoferrin, Elastase, Lysozyme, other unknown antigens |
| Autoimmune hepatitis | pANCA, atypical ANCA | |

cANCA exhibit a high sensitivity and specificity for Wegener's granulomatosis (prevalence 80 to 95%). The antibody titre correlates with the disease activity. Moreover, cANCA can occur in rare cases in microscopic arteritis and polyarteritis nodosa. The main target antigen of cANCA is proteinase 3, but the occurrence of other target antigens (e.g. BPI) is discussed.
Autoantibodies against lactoferrin, myeloperoxidase, elastase and cathepsin G are associated with various autoimmune diseases like systemic lupus erythematodes, rheumatoid arthritis, Felty's syndrome, rapid progressive glomerulonephritis, microscopic polyarteritis, polyarteritis nodosa, Crohn's disease and ulcerative colitis.
Autoantibodies against BPI were described in various diseases among them Wegener’s granulomatosis, microscopic arteritis, colitis ulcerosa, Crohn’s disease, vasculitis, autoimmune hepatitis.

Antibodies against lactoferrin e.g. has been found in 45% of patients with rheumatoid arthritis with a concurrent vasculitis. 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. Antibodies to neutrophil elastase are rarely detected, e.g. in systemic lupus erythematosus with a prevalence of 6%. The further clinical significance of these autoantibodies is yet insufficiently investigated.

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