EUROLINE Anti-MPO, -PR3 and -GBM (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
DL 1200-1601-3 G DL 1200-6401-3 G	MPO, PR3 and GBM	IgG	Ag-coated immunoblot strips	16 x 01 (16) 64 x 01 (64)

Indications: The EUROLINE test kit provides a qualitative in vitro assay for human antibodies of the immunoglobulin class IgG against the 3 different antigens **myeloperoxidase (MPO), proteinase 3 (PR3) and glomerular basement membrane (GBM antigens)** in serum or plasma for the diagnosis of granulomatosis with polyangiitis (Wegener's) (GPA), microscopic polyarteritis and Goodpasture syndrome.

Application: The EUROLINE for the determination of antibodies against myeloperoxidase, proteinase 3 (IgG) and glomerular basal membrane offers a multiplex approach to the determination of these antibodies in one reaction, with optional fully automated processing and objective evaluation of test results using the EUROLineScan software. For ANCA diagnostics, the International Consensus Statement on Testing and Reporting of Antineutrophil Cytoplasmic Antibodies (ANCA) recommends the combination of indirect immunofluorescence and mono-specific anti-myeloperoxidase (MPO), as well as anti-proteinase 3 (PR3) test systems.

Principles of the test: The test kit contains test strips coated with parallel lines of highly purified antigens. In the first reaction step, the immunoblot strips are incubated with diluted patient samples. In the case of positive samples, the specific IgG antibodies (also IgA and IgM) will bind to the corresponding antigenic site. 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	Format	Format	Symbol
1. Test strips coated with the antigens: MPO, PR3 and GBM	16 strips	4 x 16 strips	STRIPS
2. Positive control (IgG, human), 100x concentrate	1 x 0.02 ml	4 x 0.02 ml	POS CONTROL 100x
3. Enzyme conjugate Alkaline phosphatase-labelled anti-human IgG (goat), 10x concentrate	1 x 3 ml	4 x 3 ml	CONJUGATE 10x
4. Blocking buffer ready for use	1 x 30 ml	2 x 60 ml	BLOCK BUFFER
5. Universal buffer 10x concentrate	1 x 50 ml	2 x 100 ml	BUFFER 10x
6. Substrate solution Nitroblue tetrazolium chloride/5-Bromo-4-chloro-3-indolylphosphate (NBT/BCIP), ready for use	1 x 30 ml	4 x 30 ml	SUBSTRATE
7. Incubation tray	2 x 8 channels	---	
8. Test instruction	1 booklet	1 booklet	
LOT Lot description IVD In vitro diagnostic medical device	CE		Storage temperature Unopened usable until

Storage and stability: The test kit must 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, controls and incubated test strips should be handled as infectious waste. Other reagents do not need to be collected separately, unless stated otherwise in official regulations.

The following components are not provided in the test kits but can be ordered at EUROIMMUN under the respective order numbers.
Performance of the test requires an **incubation tray**:
ZD 9895-0130 Incubation tray with 30 channels
ZD 9898-0144 Incubation tray with 44 channels (black, for the EUROBlotOne and EUROBlotCamera system)
For the creation of work protocols and the evaluation of incubated test strips using **EUROLineScan** green paper and adhesive foil are required:
ZD 9880-0101 Green paper (1 sheet)
ZD 9885-0116 Adhesive foil for approx. 16 test strips
ZD 9885-0130 Adhesive foil for approx. 30 test strips
If a visual evaluation is to be performed in individual cases, the required evaluation protocol can be ordered under:
ZD 1200-0101-3 G Visual evaluation protocol EUROLINE Anti-MPO, -PR3 and -GBM (IgG).

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. Unopened, reagents are stable until the indicated expiry date when stored at +2°C to +8°C. After initial opening, reagents are stable for 12 months or until the expiry date, unless stated otherwise in the instructions. Opened reagents must also be stored at +2°C to +8°C and protected from contamination.

- **Coated test strips:** Ready for use. Open the package with the test strips only when the strips have reached room temperature (+18°C to +25°C) to prevent condensation on the strips. After removal of the strips the package should be sealed tightly and stored at +2°C to +8°C.

- **Positive control:** The control is a 100x concentrate. For the preparation of the ready for use control the amount required should be removed from the bottle using a clean pipette tip and diluted 1:101 with universal buffer. Example: add 15 µl of control to 1.5 ml of universal buffer and mix thoroughly. The ready for use diluted control should be used at the same working day.

- **Enzyme conjugate:** The enzyme conjugate is supplied as a 10x concentrate. For the preparation of the ready for use enzyme conjugate the amount required should be removed from the bottle using a clean pipette tip and diluted 1:10 with universal buffer. For one test strip, dilute 0.15 ml enzyme conjugate with 1.35 ml universal buffer. The ready for use diluted enzyme conjugate should be used at the same working day.

- **Blocking buffer:** Ready for use.

- **Universal buffer:** The universal buffer is supplied as a 10x concentrate. For the preparation of the ready for use universal buffer shake the bottle (50 ml). The amount required should be removed from the bottle using a clean pipette tip and diluted 1:10 with deionised or distilled water. The ready for use universal buffer is used for the dilution of patient samples, the dilution of the enzyme conjugate and the washing of the test strips. For the incubation of 1 test strip 2.0 ml buffer concentrate should be diluted with 18.0 ml deionised or distilled water. The ready for use diluted universal buffer should be used on the same working day.

- **Substrate solution:** Ready for use. Close bottle immediately after use, as the contents are sensitive to light ✳.

Warning: The control 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. When using heparin plasma as sample material the MPO antigen band may occasionally show an unspecific, very weak reaction.

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: The **patient samples** for analysis are diluted **1:101** with ready for use universal buffer using a clean pipette tip. For example, add 15 µl of sample to 1.5 ml universal buffer and mix well by vortexing. Sample pipettes are not suitable for mixing.

Incubation

Pretreat:	Remove the required amount of test strips from the package and place them each in an empty channel (Make sure that the surface of the test strips is not damaged!). The number on the test strip should be visible. Fill the channels of the incubation tray according to the number of serum samples that should be tested with 1.5 ml blocking buffer each. Incubate for **15 minutes** at room temperature (+18°C to +25°C) on a rocking shaker. Afterwards aspirate off all the liquid.
Incubate: (1st step)	Fill each channel with 1.5 ml of the diluted serum samples using a clean pipette tip. Incubate at room temperature (+18°C to +25°C) for **15 minutes** on a rocking shaker.
Wash:	Aspirate off the liquid from each channel and wash **3 x 5 minutes** each with 1.5 ml working strength universal buffer on a rocking shaker.
Incubate: (2nd step)	Pipette 1.5 ml diluted enzyme conjugate (alkaline phosphatase-labelled anti-human IgG) into each channel and incubate for **15 minutes** at room temperature (+18°C to +25°C) on a rocking shaker.
Wash:	Aspirate off the liquid from each channel. Wash as described above.
Incubate: (3rd step)	Pipette 1.5 ml substrate solution into the channels of the incubation tray. Incubate for **15 minutes** at room temperature (+18°C to +25°C) on a rocking shaker.
Stop:	Aspirate off the liquid from each channel and wash each strip **3 x 1 minute** with distilled water.
Evaluate:	Place test strip on the evaluation protocol, air dry and evaluate.

For automated incubation with the **EUROBlotMaster** select the program **Euro04 ANCA EL15**.

For automated incubation with the **EUROBlotOne** select the program **Euro04**.

EUROLINE Anti-MPO, -PR3, -GBM (IgG)

Incubation protocol

Pretreat
Put the test strip into the incubation channel and fill each channel with 1.5 ml blocking buffer

15 min — Shake

1. Step: Incubate
Aspirate off, pipette 1.5 ml of diluted sample (1:101) into the incubation channel

15 min — Shake

Wash
Aspirate off, wash 3 x 5 min with 1.5 ml working strength universal buffer each

2. Step: Incubate
Aspirate off, pipette 1.5 ml enzyme conjugate into the incubation channel

15 min — Shake

Wash
Aspirate off, wash 3 x 5 min with 1.5 ml working strength universal buffer each

3. Step: Incubate
Aspirate off, pipette 1.5 ml substrate into the incubation channel

15 min — Shake

Stop
Aspirate off, rinse three times with 1.5 ml distilled water

Evaluation
EUROLineScan (digital)

Interpretation of results

Handling: For the evaluation of incubated test strips we generally recommend using the **EUROLineScan** software. After stopping the reaction using deionised or distilled water, place the incubated test strips onto the adhesive foil of the green work protocol using a pair of tweezers. The position of the test strips can be corrected while they are wet. As soon as all test strips have been placed onto the protocol, they should be pressed hard using filter paper and left to air-dry. After they have dried, the test strips will be stuck to the adhesive foil. The dry test strips are then scanned using a flatbed scanner (EUROIMMUN AG) and evaluated with **EUROLineScan**. Alternatively, imaging and evaluation is possible directly from the incubation trays (EUROBlotCamera and EUROBlotOne). For general information about the EUROLineScan program please refer to the EUROLineScan user manual (YG_0006_A_UK_CXX, EUROIMMUN AG). The code for entering the **test** into EUROLineScan is **MPG**.

If a visual evaluation must be performed, place the incubated test strips onto the respective work protocol for visual evaluation. This protocol is available at EUROIMMUN under the order no. ZD 1200-0101-3 G.

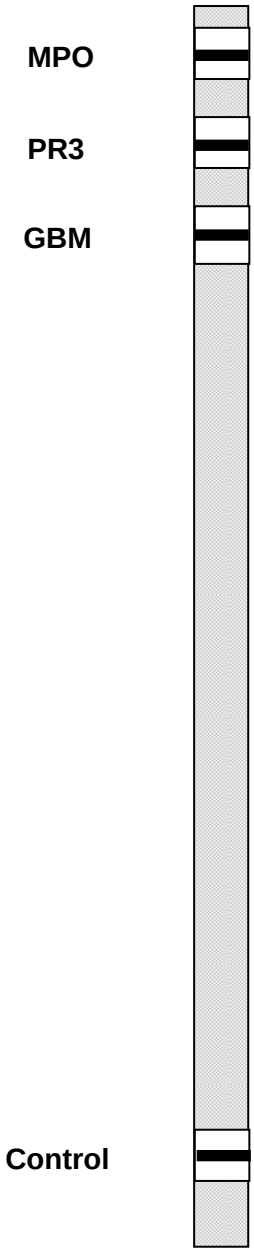
Note: Correct performance of the incubation is indicated by an intense staining of the control band.

Antigens and their arrangement on the strips: The EUROLINE test strips have been coated with the following antigens:

MPO: Native myeloperoxidase, purified from human neutrophil granulocytes.

PR3: Native proteinase 3, purified from human neutrophil granulocytes.

GBM: Native alpha-3-chains of collagen type IV which contain the relevant antigenic epitopes of the GBM antigen, purified from bovine kidney.



EUROIMMUN recommends interpreting results based on the signal intensity:

Signal **Visual evaluation**	**Signal intensity** **EUROLineScan** **Flatbed scanner**	**Result**	
No signal	0-5	o	Negative
Very weak band	6-10	(+)	Borderline
Medium to strong band	11-25 or 26-50	+, ++	Positive
Very strong band with an intensity comparable to the control band	>50	+++	Strong positive

Results in the **borderline range** (+) should be evaluated as increased but negative. The table above contains **values** for the evaluation using a flatbed scanner. The **values** for other instruments supported by EUROLineScan can be found in the EUROLineScan program. To do so mark the corresponding assay in the test list (main menu: "Help" → "Test") and click on details and select **the corresponding instrument** in **"image source"**.

An indirect immunofluorescence test to detect ANCA should always be performed in parallel with the determination of antibodies against MPO and PR3 by EUROLINE. On the one hand, this provides a check on plausibility as a safeguard against false-positive and false-negative results, on the other hand, using the **EUROIMMUN Granulocyte Mosaic immunofluorescence test** permits the detection of a wider range of ANCA, as only MPO and PR3 are presently available in the EUROLINE.

For diagnosis, the clinical picture of the patient always needs to be taken into account along with the serological findings.

Test characteristics

Antigens: The cytoplasmic granules of the granulocytes contain lots of proteins, among them proteinase 3, lactoferrin, myeloperoxidase, elastase, cathepsin G and lysozyme. **Myeloperoxidase** (molecular mass 118 kDa) is involved in the production of oxygen radicals (O_2^-, H_2O_2, OCl^-) which are toxic for many bacteria.
Proteinase 3 is a neutral serine protease which can degrade connective tissue proteins like elastin, fibronectin and collagen type IV. The enzyme exists in 3 isoforms of which the main band shows a molecular mass of 26.8 kDa in the SDS gel electrophoresis.
Main components of the **glomerular basement membrane (GBM)** are the extracellular matrix proteins collagen type IV, laminin, fibronectin and proteoglycans. Autoantibodies against the glomerular basement membrane target epitopes on the collagen type IV. The collagen type IV molecule consists of 3 alpha-chains with molecular masses of 170 kDa each. The alpha-chains form several triple helical domains separated by sequences incompatible with helix formation. A compact helical zone (7S domain) is located at the amino-terminal end and a globular knob (NC1 domain) at the carboxy-terminal end. The major antigen of anti-GBM antibodies is the NC1 domain of the alpha-3(IV)-chain.

Measurement range: The EUROLINE is a qualitative method. No measurement range is provided.

Cross reactions: The high analytical specificity of the test system is guaranteed by the quality of the antigen substrates used (antigens and antigen sources). This EUROLINE specifically detects IgG class antibodies to MPO, PR3 and GBM.

Interference: Haemolytic, lipaemic and icteric sera up to a concentration of 5 mg/ml for haemoglobin, of 20 mg/ml for triglycerides and of 0.4 mg/ml bilirubin showed no effect on the analytical results of the present EUROLINE.

Inter- and intra-assay variation: The inter-assay variation was determined by multiple analyses of characterised samples over several days. The intra-assay variation was determined by multiple analyses of characterised samples on one day. In every case, the intensity of the bands was within the specified range. This EUROLINE displays excellent inter- and intra-assay reproducibility.

Sensitivity and specificity: In comparison with the reference method ELISA, the investigation of sera from 113 patients with Wegener's disease or microscopic polyangiitis yielded a sensitivity of 96.1% with a specificity of 93.5%, and in comparison with the reference method indirect immunofluorescence (with n = 112), a sensitivity of 98% with a specificity of 93.7% for the detection of autoantibodies against MPO. For the detection of antibodies against PR3, the mentioned reference methods yielded the following sensitivities and specificities for the same patient panel. ELISA: 92.6%/98.3%; indirect immunofluorescence: 87.9%/100%. With respect to the reference method indirect immunofluorescence, the examination of 22 patient sera (12 sera from patients with Goodpasture syndrome and 10 sera from patients with suspected Goodpasture syndrome) showed a sensitivity of 82% for the determination of GBM antibodies. The examination of a control group (n = 75) of sera with antibodies against nuclear antigens (ANA) from a reference laboratory (n = 30) and of sera from SLE patients (n = 45) resulted in a specificity of 100% for the determination of GBM antibodies.

n = 113		**Anti-MPO-ELISA (IgG)**	
		positive	negative
EUROLINE Anti-MPO, -PR3 and -GBM (IgG)	positive	49	4
	negative	2	58

n = 112		**IIFT pANCA**	
		positive	negative
EUROLINE Anti-MPO, -PR3 and -GBM (IgG)	positive	48	4
	negative	1	59

n = 113		**Anti-PR3 ELISA (IgG)**	
		positive	negative
EUROLINE Anti-MPO, -PR3 and -GBM (IgG)	positive	50	1
	negative	4	58

n = 113		**IIFT cANCA**	
		positive	negative
EUROLINE Anti-MPO, -PR3 and -GBM (IgG)	positive	51	0
	negative	7	55

Reference range: The reference range was determined using a cohort of healthy blood donors (n = 61). All blood donors were negative in this EUROLINE.

Clinical significance

Anti-neutrophil cytoplasmic antibodies (ANCA) are autoantibodies against antigens localised predominantly in the cytoplasmic granules of neutrophils and monocytes. ANCA showing a granular fluorescence in IIFT that is evenly spread over the entire cytoplasm of the granulocytes, excepting the nuclei, are called cANCA (cytoplasmic pattern). Those which produce a predominantly smooth, partly fine granular fluorescence wrapped ribbon-like around the cell nuclei of the granulocytes are known as pANCA (perinuclear pattern). Atypical cANCA and pANCA fluorescence patterns are possible.

ANCA-associated antigens include
- **MPO** (myeloperoxidase), mostly the pANCA-associated enzyme,
- **PR3** (proteinase 3, myeloblastin), mostly the cANCA-associated enzyme.

ANCA-associated autoimmune diseases/small-vessel vasculitides include
- Granulomatosis with polyangiitis (Wegener's) (GPA), previously Wegener's granulomatosis (WG),
- microscopic polyangiitis (MPA),
- eosinophilic granulomatosis with polyangiitis (EGPA), previously Churg-Strauss syndrome (CSS),
- immune-complex vasculitides
  - anti-basement membrane glomerulonephritis (anti-GBM glomerulonephritis)
  - Goodpasture syndrome (rapidly progressive anti-GBM glomerulonephritis with lung bleeding).

ANCA-associated vasculitides are characterised by poor blood supply to organs due to necrotising inflammation of the vessels, the formation of microneurysms and bleeding of destroyed blood vessels. Imaging and histological diagnostic methods for the detection or exclusion of small-vessel vasculitis and the assessment of the inflammatory activity or disease course do not give meaningful results. For this reason, qualitative and quantitative serological ANCA detection plays a decisive role for diagnosis. At the beginning of the disease, the ANCA titer is generally high and decreases during treatment. It can rise again later, but this is not necessarily associated with a relapse.

Granulomatosis with polyangiitis (Wegener's) (GPA), a systemic disease of the vascular system with granuloma formation, is found in around 5 to 7 people in 100.000 mostly between 60 and 70 years of age. The disease manifests mainly in the ear, nose and throat area (approx. 85%), the lungs (approx. 65%) and the kidneys (approx. 90%).

Serological detection: cANCA, highly specific in most GPA patients. The prevalence of these antibodies in GPA with glomerulonephritis is more than 90%. In GPA without glomerulonephritis it is around 70%, in remission 30% to 40%. Classical cANCA are almost always directed against PR3, and very rarely against MPO or against both PR3 and MPO simultaneously.

Microscopic polyangiitis (MPA) is closely related to GPA but without granuloma formation. The upper respiratory tract is rarely affected. The disease leads to vasculitides of the kidneys with progressive glomerulonephritis and renal hypertonia in around 70%, and vasculitis of the lungs and skin with purpura and necrosis, predominantly at the lower extremities in around 40% of cases. Additional symptoms are polyneuritis, sinusitis, episcleritis, myalgia and arthralgia.

Serological detection: pANCA with the enzyme MPO as the main target antigen (prevalence 40% to 80%); cANCA (prevalence around 30%).

Eosinophilic granulomatosis with polyangiitis (EGPA) is characterised by a hard-to-control asthmatic and vasculitic component (asthma in 100% and rhinitis/sinusitis in around 70% of cases). The vascular changes are mainly found in the skin, nervous system and heart. The main cause of death is eosinophilic granulomatous myocarditis.

Serological detection: pANCA with MPO as the main target antigen; prevalence 40% to 70%.

Anti-GBM glomerulonephritis accounts for 0.5% to 2% of all cases of glomerulonephritis.

Serological detection: In cases without involvement of the lungs the prevalence of autoantibodies against the extracellular matrix protein of the glomerular basement membrane (anti-GBM antibodies) is 30% to 60%, and with lung involvement 80% to 90%. Therefore, antibodies against GBM should be investigated in all illnesses with deterioration of kidney function.

Goodpasture's syndrome, an anti-GBM glomerulonephritis with lung involvement, is characterised by bloody secretion, which leads to lung siderosis. Without treatment, Goodpasture's syndrome has a very poor prognosis. Since the disease progresses rapidly, early diagnosis is imperative and can be confirmed by the detection of anti-GBM antibodies.

Serological detection: Prevalence of anti-GBM antibodies of 80% to 90%; of ANCA 10% to 30%.

Note:

MPO is the main antigen of pANCA in patients with MPA and EGPA. However, pANCA can also occur in patients without vasculitis, e.g. in inflammatory bowel diseases, primary sclerosing cholangitis, autoimmune liver diseases, collagenoses, rheumatoid arthritis, malignant tumours and infections. In these disorders, ANCA are almost always directed against neutrophil constituents besides MPO.

Further target antigens of pANCA that have been identified are as follows: lactoferrin, elastase, BPI, cathepsin G, lysozyme and β-glucuronidase.

The combination of IIFT with Anti-PR3 ELISA and Anti-MPO ELISA or Anti-MPO, Anti-PR3 and Anti-GBM immunoblot for IgG (EUROLINE) showed an extremely high specificity of 99% for the diagnosis of small-vessel vasculitis.

In parallel to determining antibodies against MPO, PR3 and GBM (EUROLINE), IIFT for the detection of ANCA and anti-GBM should always be performed. This enables verification of plausibility, for example to safeguard against false positive and false negative results. Moreover, IIFT allows a wider spectrum of ANCA to be detected.

In the serological diagnosis of the chronic inflammatory bowel diseases ulcerative colitis and Crohn's disease, the detection of pANCA (IgA and IgG) in combination with the detection of autoantibodies against exocrine pancreas and/or autoantibodies against intestinal goblet cells plays an important role.

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