Granulocyte Mosaic and EUROPLUS Granulocyte Mosaic
Instructions for the indirect immunofluorescence test

ORDER NO.	ANTIBODIES AGAINST	SUBSTRATE	SPECIES	FORMAT SLIDES x FIELDS
FA 1200 to FA 1202 (see p. 16)	cANCA, pANCA	granulocytes (EOH)	human	10 x 03 (030)
	cANCA, pANCA	granulocytes (HCHO)	human	10 x 05 (050)
	cANCA, pANCA	granulocytes (MOH)	human	10 x 10 (100)
	myeloperoxidase	MPO EUROPLUS	---	10 x 50 (500)
	proteinase 3	PR3 EUROPLUS	---	20 x 05 (100)
	glomerular basement membrane (GBM)	GBM EUROPLUS	---	20 x 10 (200)
	DNA-bound lactoferrin	lactoferrin-specific granulocytes	human	24 x 50 (1200)
	---	HSS granulocytes (high-salt stripped)	human	120 x 10 (1200)
	cell nuclei (ANA)	HEp-2 cells	human	180 x 10 (1800)
	cell nuclei (ANA), cANCA, pANCA	HEp-2 cells + granulocytes (EOH)	human	

Indication: This test kit provides qualitative or semiquantitative in vitro determination of human antibodies of immunoglobulin class IgG against granulocyte cytoplasm (ANCA) in patient samples to support the diagnosis of the following diseases: granulomatosis with polyangiitis (GPA, Wegener's granulomatosis), eosinophile granulomatosis with polyangiitis (EGPA, Churg-Strauss syndrome), microscopic polyangiitis (MPA), primary sclerosing cholangitis and chronic inflammatory bowel diseases (mainly ulcerative colitis).

Application: Following an internationally valid directive (International Consensus Statement), the indirect immunofluorescence test is considered the gold standard for the detection of antibodies against neutrophil granulocytes. Positive IFT results need to be confirmed with a monospecific anti-PR3, anti-MPO or anti-GBM test. The EUROPLUS technique enables the combination of conventional cell culture substrates with defined individual antigens in one test field. This simplifies the interpretation of the immunofluorescence patterns of pANCA, cANCA and ANCA against further target antigens (e.g. DNA-ANCA) considerably. Evaluation can take place immediately after the first incubation, as the results of the screening test (granulocytes) are confirmed antigen-specifically in the same test run. For quantification of results, we recommend to perform the corresponding ELISA afterwards.

Test principle: Combinations of substrates are incubated with diluted patient samples. If the reaction is positive, specific antibodies of classes IgA, IgG and IgM attach to the antigens. In a second step, the bound antibodies are stained with FITC-labelled anti-human antibodies and made visible with the fluorescence microscope.

Contents of a test kit for 50 determinations (e.g. FA 1201-1005-13):

Description	Format	Symbol
1. Slide, each containing 5 x 3 BIOCHIPs coated with granulocytes (EOH), HEp-2 cells + granulocytes (EOH) and granulocytes (HCHO)	10 slides	SLIDE
2. FITC-labelled anti-human IgG (goat), ready for use	1 x 1.5 ml	CONJUGATE
3. Positive control with titer information: autoantibodies against granulocyte cytoplasm (cANCA), human, ready for use	1 x 0.1 ml	POS CONTROL
4. Positive control with titer information: autoantibodies against myeloperoxidase (anti-MPO), human, ready for use	1 x 0.1 ml	POS CONTROL
5. Negative control: autoantibody negative, human, ready for use	1 x 0.1 ml	NEG CONTROL
6. Salt for PBS pH 7.2	2 packs	PBS
7. Tween 20	2 x 2.0 ml	TWEEN 20
8. Mounting medium, ready for use	1 x 3.0 ml	GLYCEROL
9. Cover glasses (62 mm x 23 mm)	12 pieces	COVERGLASS
10. Instruction booklet	1 booklet	---

LOT	Lot description		Storage temperature
IVD	In vitro diagnostic medical device	CE	Unopened usable until

Modifications to the former version are marked in grey.

Single slides (e.g., EUROIMMUN order no. FB 1201-1005-13) are provided together with cover glasses. Additional positive control (e.g., order no. CA 1200-0101-3, CA 1211-0101-3) and negative control (e.g., order no. CA 1000-0101) can be ordered.
Performance of the test requires reagent trays TRAY, which are not provided in the test kits. They are available from EUROIMMUN under the following order no.:
- ZZ 9999-0110 Reagent trays for slides containing up to 10 fields.

Performing the test (reaction fields 5 x 5 mm)

The **TITERPLANE Technique** was developed by EUROIMMUN in order to standardise immunological analyses: Samples or labelled antibodies are applied to the reaction fields of a reagent tray. The BIOCHIP slides are then placed into the recesses of the reagent tray, where all BIOCHIPs of the slide come into contact with the fluids, and the individual reactions commence simultaneously. Position and height of the droplets are exactly defined by the geometry of the system. As the fluids are confined to a closed space, there is no need to use a conventional "humidity chamber". It is possible to incubate any number of samples next to each other and simultaneously under identical conditions.

Prepare: The preparation of the reagents and of the serum and plasma samples is described on **page 4** of this test instruction.

Pipette: Apply **30 µl of diluted sample** to each reaction field of the reagent tray, avoiding air bubbles. Transfer all samples to be tested before starting the incubation (up to 200 droplets). Use a polystyrene pipetting template.

Incubate: Start reactions by fitting the BIOCHIP slides into the corresponding recesses of the reagent tray. Ensure that each sample makes contact with its BIOCHIP and that the individual samples do not come into contact with each other.
Incubate for **30 minutes** at room temperature (+18°C to +25°C).

Wash: Rinse the BIOCHIP slides with a flush of PBS-Tween using a beaker and immerse them immediately afterwards in a cuvette containing PBS-Tween for at least **5 minutes**. Shake with a rotary shaker if available. Wash max. 16 slides, then replace PBS-Tween with new buffer.

Pipette: Apply **25 µl of conjugate** to each reaction field of a clean reagent tray. Add all droplets before continuing incubation. Use a stepper pipette. The conjugate should be mixed thoroughly before use. To save time, conjugate can be pipetted onto separate reagent trays during the incubation with the diluted sample.

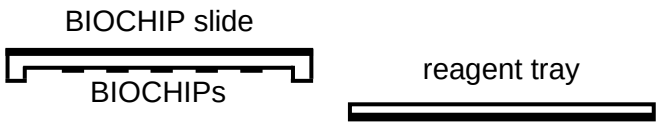
Incubate: Remove one BIOCHIP slide from the cuvette. Within five seconds blot only the back and the long sides with a paper towel and immediately put the BIOCHIP slide into the recesses of the reagent tray. Do not dry the areas between the reaction fields. Check for correct contact between the BIOCHIPs and liquids. Then continue with the next BIOCHIP slide. From now on, protect the slides from direct sunlight.
Incubate for **30 minutes** at room temperature (+18°C to +25°C).

Wash: Fill cuvette with new PBS-Tween. Rinse the BIOCHIP slides with a flush of PBS-Tween using a beaker and put them into the cuvette filled with the new PBS-Tween for at least **5 minutes**. Shake with a rotary shaker if available. Wash max. 16 slides, then replace PBS-Tween with new buffer.

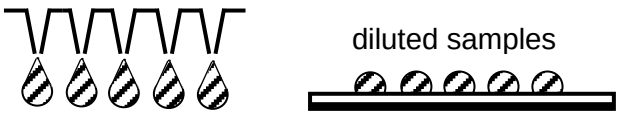
Mount: Place mounting medium onto a cover glass – drops of **max. 10 µl per reaction field**. Use a polystyrene mounting tray. Remove one BIOCHIP slide from PBS-Tween and dry the back and all four sides with a paper towel. Put the BIOCHIP slide, with the BIOCHIPs facing downwards, onto the prepared cover glass. Check immediately that the cover glass is properly fitted into the recesses of the slide. Correct the position if necessary.

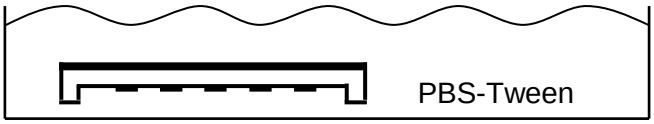
Evaluate: Read the fluorescence with the microscope.
General recommendation: objective 20x (tissue sections, infected and transfected cells), 40x (cell substrates).
Excitation filter: 450-490 nm, colour separator: 510 nm, blocking filter: 515 nm.
Light source: mercury vapour lamp, 100 W, EUROIMMUN LED, EUROStar Bluelight.

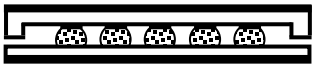
TITERPLANE Technique

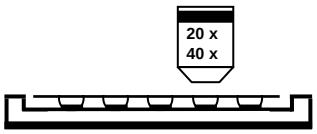


Pipette:	30 µl per field
Incubate:	30 min
Wash:	1 s flush 5 min cuvette
Pipette:	25 µl per field
Incubate:	30 min
Wash:	1 s flush 5 min cuvette
Mount:	max. 10 µl per field
Evaluate:	fluorescence microscopy









Automated Incubation: The test kit can be incubated by using automated devices, e.g. IF Sprinter, Sprinter XL, EUROLabLiquidHandler or others. The incubation and washing conditions programmed should be the same as described in the manual procedure. The test settings for EUROIMMUN devices are validated in combination with the kit. Any other combination has to be validated by the user. For details please refer to the device manual.

Preparation and stability of reagents

Note: After initial opening, the reagents are stable until the expiry date when stored between +2°C and +8°C and protected from contamination, unless stated otherwise below.

- **Slides:** Ready for use. Remove the protective cover only when the slides have reached room temperature (+18°C up to +25°C); condensed water can damage the substrate). Do not touch the BIOCHIPs. If the protective cover is damaged, the slide must not be used for diagnostics.

- **FITC-labelled secondary antibody:** Ready for use. Before using for the first time, mix thoroughly. The conjugate is sensitive to light. Protect from sunlight ✳.

- **Positive and negative controls:** Ready for use. Before using for the first time, mix thoroughly. Positive controls with titer information can be used both diluted and undiluted for titer determination.
  Positive control with titer information: The label contains the target value and the substrate used to determine the target value. The lower tolerance limit is one titer level below the target value, the upper tolerance limit lies two titer levels above the target value. The control is to be diluted with PBS-Tween. Diluted controls must be incubated within one working day.

- **PBS-Tween:** 1 pack of “Salt for PBS” should be dissolved in 1 liter of distilled water (optimal: aqua pro infusione, aqua ad injectabilia) and mixed with 2 ml of Tween 20 (stir for 20 minutes until homogeneous). The prepared PBS-Tween can be stored at +2°C to +8°C, generally for 1 week. PBS-Tween should not be used if the solution becomes cloudy or contamination appears.

- **Mounting medium:** Ready for use.

- **Reagent trays:** Reaction fields of the reagent tray must be hydrophilic and surrounding area hydrophobic. If necessary, leave in 2% Deconex 11 universal (EUROIMMUN order number: ZZ 9912-0101) for 12 hours. Afterwards rinse generously with water and dry. Cleaning: Rub reagent trays with 5% Extran MA 01 (EUROIMMUN order number: ZZ 9911-0130) and rinse with plenty of with water. To disinfect: Spray reagent trays generously with Mikrozid AF (EUROIMMUN order number: ZZ 9921-0125), turn over and leave for 5 minutes. Afterwards, rinse generously with water and dry.

Storage and stability: The slides and the reagents should be stored at a temperature between +2°C and +8°C. Unopened, all test kit components are stable until the indicated expiry date.

Waste disposal: Patient samples, controls and slides are to be handled as potentially infectious materials. All reagents are to be disposed of in accordance with official disposal regulations.

Warning: The BIOCHIPs coated with antigen substrates have been treated with a disinfecting fixing agent. Neither HBsAg nor antibodies against HIV-1, HIV-2, and HCV could be detected in the control sera using appropriate ELISA or indirect immunofluorescence tests. **Nevertheless, all test system components should be handled as potentially infectious materials.** Some of the reagents also contain sodium azide in a non-declarable concentration. Avoid skin contact.

Preparation and stability of patient samples

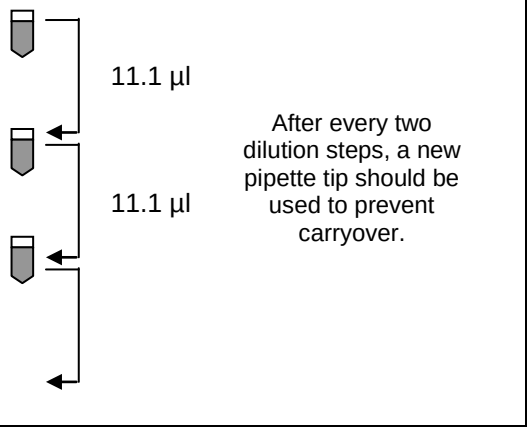
Samples: Human sera or EDTA, heparin or citrate plasma.

Stability: The patient samples to be investigated can generally be stored for up to 14 days at a temperature between +2°C and +8°C. Diluted samples must be incubated within one working day.

Recommended sample dilution for qualitative evaluation: The sample to be investigated is diluted 1:10 in PBS-Tween. For example, dilute 11.1 µl sample in 100 µl PBS-Tween and mix thoroughly, e.g. vortex for 4 seconds.

Recommended sample dilution for semiquantitative evaluation: The dilution of samples to be investigated is performed using PBS-Tween. Add 100 µl of PBS-Tween to each tube and mix with 11.1 µl of the next highest concentration, e.g. vortex for 2 seconds. EUROIMMUN recommends incubating samples from a dilution of 1:10.

Dilution	Dilution scheme
1:10	100 µl PBS-Tween + 11.1 µl undiluted sample
1:100	100 µl PBS-Tween + 11.1 µl 1:10 diluted sample
1:1000	100 µl PBS-Tween + 11.1 µl 1:100 diluted sample
⋮	⋮



Test evaluation

Fluorescence pattern (positive reaction): In the case of autoantibodies against the cytoplasm of **granulocytes fixed with ethanol**, two relevant 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**: anti-neutrophilic cytoplasm antibodies, cytoplasmic pattern) is caused by antibodies against proteinase 3 localised in the azurophilic granula of the neutrophils. However, a cANCA fluorescence can also appear if antibodies against bactericidal permeability increasing protein (BPI, CAP57) are present. In exceptional cases the same pattern occurs due to antibodies against myeloperoxidase, which otherwise show a pANCA pattern. A smooth fluorescence wrapped ribbon-like around the cell nuclei of the granulocytes (**pANCA**: anti-neutrophilic cytoplasm antibodies, perinuclear pattern; more or less extended) can be caused by antibodies of various specificities. The ribbon-like, perinuclear fluorescence of pANCA results from the fact that during incubation with the antibodies of the patient serum the antigens diffuse out of the granula to the nuclear membrane, to which they have a high affinity. Target antigens of pANCA are myeloperoxidase, granulocyte-specific elastase, DNA-bound lactoferrin, lysozyme, cathepsin G, beta-glucuronidase, azurocidin, h-lamp-2, alpha-enolase, defensin and other, partly not yet identified antigens.

The advantage of immunofluorescence using granulocyte smears as substrate compared with a monospecific ELISA is that it can at the same time detect antibodies against all of these antigens and against further, as yet unknown antigens with both high sensitivity and specificity. Some of the granulocytes (eosinophils, basophils) do not react.

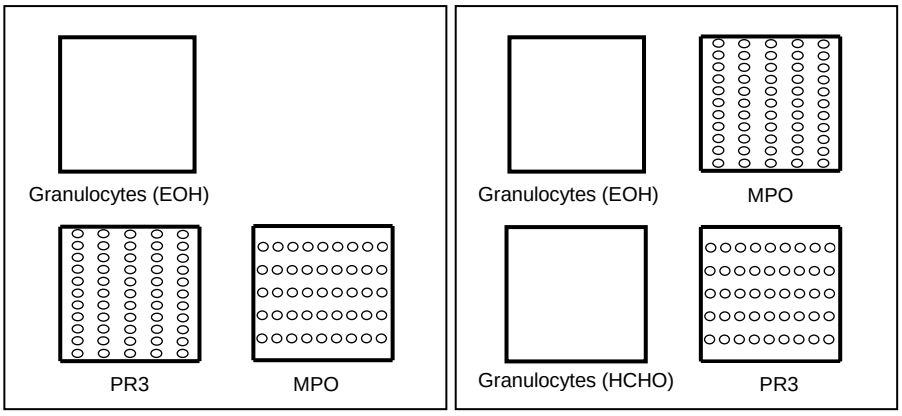
In the case of autoantibodies against the cytoplasm of granulocytes, one relevant fluorescence pattern can be detected with **formaldehyde-fixed granulocytes**, i.e. a granular fluorescence which is distributed regularly over the entire cytoplasm of the granulocytes, leaving the cell nuclei free. This pattern is particularly caused by antibodies against proteinase 3 (**cANCA**: anti-neutrophilic cytoplasm antibodies, cytoplasmic type) localised in the azurophilic granula of the neutrophils and autoantibodies against myeloperoxidase (**pANCA, MPO**). Normally, formaldehyde-fixed granulocytes do not react with antibodies against elastase, lactoferrin, lysozyme, beta-glucuronidase and cathepsin G and they hardly ever show a reaction with antibodies against cell nuclei. Some of the granulocytes (eosinophils, basophils) do not fluoresce.

Two fluorescence patterns can be detected with **methanol-fixed granulocytes**: a granular fluorescence which is distributed regularly over the entire cytoplasm of the neutrophils, leaving the cell nuclei free (**cANCA**, proteinase 3) and a mainly smooth fluorescence wrapped ribbon-like around the cell nuclei (perinuclear type, **pANCA**). Antibodies directed against myeloperoxidase (**pANCA, MPO**) react weakly or not at all with methanol-fixed granulocytes. Some of the granulocytes (eosinophils, basophils) do not fluoresce.

If the sera are negative, the granulocytes are dark. A granular cytoplasmic fluorescence of a small percentage of the granulocytes (eosinophils, basophils) is generally without significance. With ethanol-fixed granulocytes, some antibodies against nuclear antigens also react. However, this does not apply to antibodies against mitochondria. A granular fluorescence of the cell nuclei should not be mixed up with cANCA.

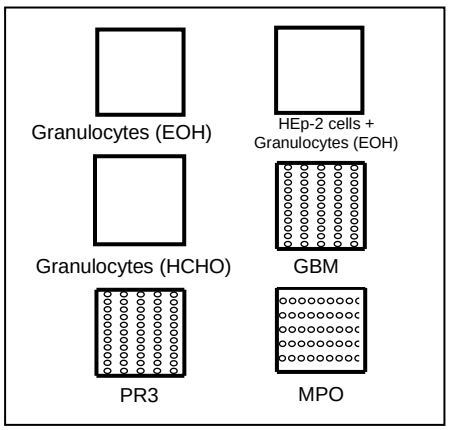
Antibodies against nuclear antigens **(ANA)** can be found on numerous substrates. For the targeted determination and differentiation of antinuclear antibodies, **human epithelial cells (HEp-2 cells)** are used as the substrate. The cell nuclei show a distinct fluorescence, which is characterised by certain patterns. In the case of negative samples, the nuclei show no specific fluorescence. With the combination BIOCHIP HEp-2 cells + granulocytes (EOH), antibodies against cell nuclei and granulocyte cytoplasm can be detected in the same microscopic field of vision.

Arrangement of BIOCHIPs on the fields of the different mosaics (see page 16):

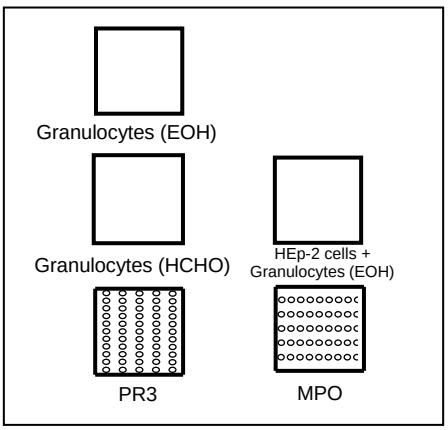


FA 1201-####-20

FA 1201-####-22



FA 1201-####-25



FA 1201-####-32

The BIOCHIPs of the **EUROPLUS** substrates **MPO, PR3 and GBM** are coated with microscopically small droplets of highly purified antigens.
If a sample contains antibodies which are directed against these antigens, the respective antigen dots fluoresce as green circular areas on a dark background.

Antibodies against **DNA-bound lactoferrin** cause a nuclear fluorescence of the granulocyte nuclei on granulocytes selectively reacting with lactoferrin. The HSS granulocytes incubated in the same reaction field must be negative or show a significantly weaker reaction. The same pattern must essentially be obtained as for the positive control serum.
If both substrates show a comparable fluorescence or the HSS granulocytes fluoresce more than the granulocyte nuclei, it is highly probable that nuclear antibodies (e.g. antibodies against dsDNA, ssDNA, nucleosomes or histones) are present in the patient sample. In this rare case the result should not be evaluated.

In a **negative reaction** the entire BIOCHIP remains dark and the described circular areas can hardly be detected or not detected at all. For a secure differentiation between positive and negative results the positive and negative control and some normal sera, if necessary, must be compared with the patient samples.

If the positive control shows no specific fluorescence pattern or the negative control shows a clear specific fluorescence, the results are not to be used and the test is to be repeated.

A large range of fluorescence images can be found on the EUROIMMUN website (www.euroimmun.com).

Recommended qualitative evaluation:

ANCA reactivity (IgG)	**Evaluation**
No reaction at 1:10	Negative. No IgG class antibodies against the cytoplasm of granulocytes detected in the patient sample.
Positive reaction at 1:10	Positive. Indication of Wegener's granulomatosis (cANCA, PR3), microscopic polyangiitis (pANCA, MPO), ulcerative colitis (pANCA, formalin-sensitive) and some other ANCA-associated diseases.

The substrates HEp-2 cells and HEp-2 cells + granulocytes (EOH) are used in this combination only for the purpose to differentiate between pANCA and ANA. A positive result for ANA with a titer of 1:<100 is not to be evaluated.

Anti-GBM reactivity (IgG)	**Evaluation**
No reaction at 1:10	Negative. No antibodies against GBM detected in the patient sample.
Positive reaction at 1:10	Positive. Indication of glomerulonephritis, Goodpasture's syndrome.

Anti-DNA-bound lactoferrin reactivity (IgG)	**Evaluation**
No reaction at 1:10	Negative. No antibodies against DNA-bound lactoferrin detected in the patient sample.
Positive reaction at 1:10	Positive. If corresponding symptoms are present, indication of chronic-inflammatory intestinal diseases (particularly ulcerative colitis) or primary sclerosing cholangitis.

Recommended semiquantitative evaluation: The titer is defined as the sample dilution factor for which specific fluorescence is just identifiable. This should be compared with the reaction obtained using an equivalently diluted negative serum.

Antibody titers can be determined according to the following table from the fluorescence of the different sample dilutions.

Fluorescence at				Antibody titer
1:10	1:100	1:1000	1:10,000	
weak	negative	negative	negative	1:10
moderate	negative	negative	negative	1:32
strong	weak	negative	negative	1:100
strong	moderate	negative	negative	1:320
strong	strong	weak	negative	1:1000
strong	strong	moderate	negative	1:3200
strong	strong	strong	weak	1:10,000
⋮	⋮	⋮	⋮	⋮

Limitations of the procedure

1. A diagnosis should not be made based on a single test result. The clinical symptoms of the patient should always be taken into account along with the serological results by the physician.
2. Adaptation of this assay for use with automated sample processors and other liquid handling devices, in whole or in part, may yield differences in test results from those obtained using the manual procedure. It is the responsibility of each laboratory to validate that their automated procedure yields test results within acceptable limits.
3. Mishandling of slides during the staining procedure, especially allowing slides to dry between steps, may result in a “washed out" pattern appearance and/or a high level of background staining.
4. Coplin jars used for slide washing should be free from all residues. Use of coplin jars containing residues may cause staining artefacts.
5. The light source, filters and optical unit of the fluorescence microscope can influence the sensitivity of the assay. Using traditional mercury vapour lamp systems, the performance of the microscope depends on correct maintenance, especially alignment of the lamp and replacement of the lamp after the recommended period of time. The EUROIMMUN fluorescence microscopes with LED Bluelight as the light source offer many advantages. Contact EUROIMMUN for details.

Test characteristics

Antigen: Ethanol-fixed granulocytes from humans are used as a standard substrate for the detection of antibodies against granulocyte cytoplasm (cANCA, pANCA). It is often difficult to differentiate pANCA from antibodies against cell nuclei by means of indirect immunofluorescence.
For the differentiation of ANA, the combination substrate **HEp-2 cells + Granulocytes (EOH)** is mostly used alongside **HEp-2 cells**. Antibodies against cell nuclei and against granulocyte cytoplasm are detected in the same microscopic field of vision. **Formaldehyde-fixed granulocytes** enable identification of pANCA even alongside antinuclear antibodies, if they are antibodies against myeloperoxidase (MPO). Furthermore, antibodies against proteinase 3 can be made visible on formaldehyde-fixed granulocytes. The reference EUROIMMUN product is a combination of BIOCHIPs with ethanol-fixed granulocytes, formaldehyde-fixed granulocytes and HEp-2 cells + granulocytes (EOH) (“IIFT: Granulocyte Mosaic 13” EUROIMMUN order no. FA 1201-1005-13). cANCA (proteinase 3) reacts in the same way with **methanol-fixed granulocytes** as with ethanol-fixed granulocytes. Generally antibodies against MPO only show either a decreased reaction or no reaction with methanol-fixed granulocytes.

For the specific detection of autoantibodies against myeloperoxidase (MPO) and proteinase 3 (PR3) BIOCHIPs are coated with highly purified antigens isolated from neutrophile granulocytes. A supplementation of the classical ANCA screening test with granulocyte substrates with the monospecific **EUROPLUS substrates MPO and PR3** provides further diagnostic advantages: Positive results can be confirmed immediately in the same test run, unspecific or unclear fluorescence patterns can be evaluated with more certainty.
GBM BIOCHIPs are coated with recombinant GBM antigen, which corresponds to the shortened alpha-3 chain of the type IV collagen (NC-1 domain).
The indirect immunofluorescence test for the serological diagnosis of chronic-inflammatory bowel diseases uses ethanol- and formalin-fixed granulocytes. Formalin-sensitive pANCA, in particular, occur mainly in patients with ulcerative colitis (UC). Antibodies against intestinal goblet cells are only found in UC patients. Other important autoantibodies associated with ulcerative colitis are antibodies against DNA-bound lactoferrin. For the determination of these autoantibodies, granulocytes selectively reacting with lactoferrin (LFS granulocytes) are used, which were newly developed by EUROIMMUN AG.
A serological differentiation between Crohn's disease and ulcerative colitis can be achieved by the determination of antibodies against exocrine pancreas and Saccharomyces cerevisiae.
The most reliable differentiation is achieved using a substrate combination from goblet cells, rPAg, Saccharomyces cerevisiae and granulocytes (order no. FA 1391-1005-3). If granulocytes selectively reacting lactoferrin (e.g. order no. FA 1215-1005-1) are used, the serological hit rate for the diagnosis of chronic-inflammatory bowel diseases can be increased significantly.
Antibodies against DNA-bound lactoferrin can also be found in some liver diseases, especially in primary sclerosing cholangitis.

Measurement range: The dilution starting point for this measurement system is 1:10. Samples can be further diluted by a factor of 10 so that the dilution series is 1:100, 1:1000, 1:10,000 etc. There is no upper limit to the measurement range.

Reproducibility: The intensity of the specific fluorescence as a numeric value is called fluorescence intensity level by EUROIMMUN. These values can reach from "0" (no specific fluorescence) to "5" (extremely strong specific fluorescence).

Reproducibility	**Inter-lot**	**Intra-assay**	**Inter-assay**
Minimum requirement	3 lots x 3 samples x 1 run x single determination: max. ± 1 intensity level	1 lot x 3 samples x 1 run x tenfold determination: max. ± 1 intensity level	1 lot x 3 samples x 2 runs x double determination: max. ± 1 intensity level
Granulocytes (EOH)	maximum deviation ± 1 intensity level	no deviation	no deviation
Granulocytes (HCHO)	maximum deviation ± 1 intensity level	no deviation	no deviation
Granulocytes (MOH)	maximum deviation ± 1 intensity level	Is assured since inter-lot reproducibility was investigated with more than 10 lots.	Is assured since inter-lot reproducibility was investigated with more than 10 lots.
HEp-2 cells + Granulocytes (EOH)	maximum deviation ± 1 intensity level	no deviation	maximum deviation ± 1 intensity level
MPO EUROPLUS	maximum deviation ± 1 intensity level	maximum deviation ± 1 intensity level	maximum deviation ± 1 intensity level
PR3 EUROPLUS	maximum deviation ± 1 intensity level	maximum deviation ± 1 intensity level	no deviation
GBM EUROPLUS	no deviation	no deviation	no deviation
LFS granulocytes			
HEp-2	no deviation	maximum deviation ± 1 intensity level	maximum deviation ± 1 intensity level

Interference: Haemolytic, lipaemic and icteric samples showed no influences on analysis results.

Cross reactivity: Some antibodies against cell nuclei also react with ethanol-fixed granulocytes, however, antibodies against mitochondria generally do not. There were no cross reactivities between the antigens MPO and PR3. With formaldehyde-fixed granulocytes, none of the 30 ANA-positive sera reacted with an ANA-specific pattern, which can be mistaken for the specific cANCA pattern. Cross reactions with ANA are unlikely.

With HEp-2 cells, 11 characterised samples were incubated (CDC 1 to CDC 11) in which only the expected fluorescence patterns were detected. The results show that these cross reactivities are not detected.

Reference range: Titer 1:<10

The following antibody prevalences were determined using a panel of samples from healthy blood donors (origin: Germany):

Substrate	Antibodies against	Conjugate	Prevalence	Cut-off	Number of samples
Granulocytes (EOH)	cANCA, pANCA	IgA	2.9%	1:10	276
		IgG	1.7%		526
Granulocytes (HCHO)	cANCA, pANCA	IgA	0%		276
		IgG	0.4%		526
Granulocytes (MOH)	pANCA	IgG	3.0%		200
HEp-2 cells + Granulocytes (EOH)	cANCA, pANCA	IgG	3.0%		200
MPO EUROPLUS	Myeloperoxidase	IgA	0%		200
		IgG	1.0%		200
PR3 EUROPLUS	Proteinase 3	IgA	0.5%		200
		IgG	2.0%		198
GBM EUROPLUS	Glomerular basement membrane	IgG	0%		198
LFS granulocytes	DNA-bound lactoferrin	IgG	4.0%		200
HSS granulocytes	DNA-bound lactoferrin	IgG	0%		200
HEp-2	Cell nuclei (ANA)	IgG	12.5%	1:100	200

Specificity and sensitivity:

Collectives	n	IIFT cANCA	EUROPLUS Anti-PR3	Anti-PR3-hn-hr ELISA	IIFT pANCA	EUROPLUS Anti-MPO	Anti-MPO ELISA
Wegener's granulomatosis	59	47 (79.7%)	45 (76.3%)	43 (72.9%)	0	0	0
Sensitivity	**59**	**79.7%**	**76.3%**	**72.9%**	**-**	**-**	**-**
AAV, biopsy proven	112	57 (50.9%)	57 (50.9%)	57 (50.9%)	51 (45.5%)	49 (43.8%)	51 (45.5%)
AAV, outpatient	74	27 (36.5%)	26 (35.1%)	26 (35.1%)	17 (23.0%)	16 (21.6%)	11 (14.9%)
Prevalence	**186**	**45.2%**	**44.6%**	**44.6%**	**36.6%**	**34.9%**	**33.3%**
Rheumatoid arthritis	29	0	0	0	1 (3.4%)	0	1 (3.4%)
Blood donors	27	0	0	0	0	0	1 (3.7%)
Non-ANCA associated vasculitides	53	1 (1.9%)	1 (1.9%)	0	2 (3.8%)	2 (3.8%)	1 (1.9%)
Specificity collective 1	**109**	**99.1%**	**99.1%**	**100%**	**97.2%**	**98.2%**	**97.2%**
Syst. lupus erythematosus	100	4 (4.0%)	0	0	1 (1.0%)	0	n.d.
Sjögren's syndrome	196	5 (2.6%)	2 (1.0%)	1 (0.5%)	4 (2.0%)	4 (2.0%)	n.d.
Rheumatoid arthritis	200	1 (0.5%)	0	0	6 (3.0%)	5 (2.5%)	n.d.
Blood donors, asymptomatic	199	0	0	1 (0.5%)	0	0	n.d.
Specificity collective 2	**695**	**98.6%**	**99.7%**	**99.7%**	**98.4%**	**98.7%**	**-**
Specificity collective 1 + 2	**804**	**98.6%**	**99.6%**	**99.8%**	**98.3%**	**98.6%**	**-**

Substrate	Ig class	Reference (number and origin of samples)	Specificity	Sensitivity
GBM EUROPLUS: Anti-GBM	IgG	Healthy blood donors (n = 100, Germany)	100%	-
		Anti-GBM ELISA (n = 44, Germany)	-	100%
HEp-2 cells + Granulocytes (EOH)		Healthy blood donors (n = 100, Germany)	98%	--
		Anti-Granulocytes EOH IFT (n = 60, Germany)	--	100%
LFS granulocytes		Healthy blood donors (n = 200, Germany)	96%	--
HEp-2		Anti-ENA ProfilePlus ELISA and Anti-ENA ProfilePlus EUROBLOT (n = 128, Germany)	100%	100%

Substrate	Ig class	Reference (number and origin of samples)	Prevalence
LFS granulocytes	IgG	Patient group ulcerative colitis (n = 39, Germany)	71.8%
		Patient group Crohn's disease (n = 96, Germany)	6.3%

Clinical significance

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

- the enzyme myeloperoxidase (MPO), predominantly associated with pANCA,
- proteinase 3 (PR3), predominantly associated with cANCA,
- the antigen type IV collagen (extracellular matrix protein of the glomerular basement membrane (GBM).

ANCA-associated autoimmune diseases/small-vessel vasculitides include

- granulomatous polyangiitis (GPA), formerly called Wegener's granulomatosis (WG),
- microscopic polyangiitis (MPA),
- eosinophilic granulomatosis with polyangiitis (EGPA), formerly called Churg-Strauss syndrome (CSS),
- immune-complex vasculitides
    - anti-basement membrane glomerulonephritis (anti-GBM glomerulonephritis)
    - Goodpasture's 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 in the area of the 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.

Granulomatous polyangiitis (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 is almost always directed against PR3, and very rarely against MPO or against both PR3 and MPO simultaneously.

Microscopic polyangiitis (MPA) is closely related with 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%, 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%).

Eosinophile 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 eosinophile 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 antibodies against the extracellular matrix protein of the glomerular basement membrane (anti-GBM antibodies) is 30% to 60%, and with lung involvement 80% to 90%. 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 can lead 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:

Atypical cANCA IIFT patterns (flat homogeneous cytoplasmic pattern) can be associated with antibodies against BPI protein (bactericidal/permeability increasing protein), which are detectable in rare cases, e.g. Pseudomonas infections and subacute bacterial endocarditis.

MPO is the main antigen of pANCA in patients with MPA and EGPA. But pANCA can also occur in patients with 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 other than MPO.

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

With IIFT, all autoantibodies against granulocytes can be detected. The International Consensus Statement 1994 (International Chapel Hill Consensus Conference 2012) recommends screening for ANCA using IIF and the confirmation of IIFT-positive sera with both Anti-PR3 and Anti-MPO ELISA. The use of IIFT or ELISA results alone had unsatisfactory diagnostic specificity with respect to disease control sera. In contrast, 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 a 99% specificity 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 antibodies against exocrine pancreas and/or antibodies against intestinal goblet cells plays an important role.

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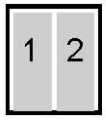
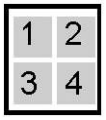
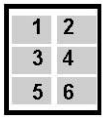
BIOCHIP position on the fields	  						
This test instruction is valid for the listed test systems (#### is a place holder for different formates, e.g. 1005 = 10 slides with 5 fields):							
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		1	2	3	4	5	6
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FA 1201-####	IIFT: Granulocytes (HCHO)	Granulocytes (HCHO)					5x5
FA 1201-####-2	IIFT: Granulocyte Mosaic 2	Granulocytes (EOH)	Granulocytes (HCHO)				5x5
FA 1201-####-4	IIFT: Granulocyte Mosaic 4	Granulocytes (EOH)	Granulocytes (HCHO)	HEp-2 cells			5x5
FA 1201-####-13	IIFT: Granulocyte Mosaic 13	Granulocytes (EOH)	---	Granulocytes (HCHO)	HEp-2 cells + Granulocytes (EOH)		5x5
FA 1201-####-15	IIFT: Granulocyte Mosaic 15	Granulocytes (EOH)	HEp-2 cells + Granulocytes (EOH)				5x5
FA 1201-####-17	IIFT: Granulocyte Mosaic 17	Granulocytes (EOH)	HEp-2 cells + Granulocytes (EOH)	Granulocytes (HCHO)	Granulocytes (MOH)		5x5
FA 1201-####-20	EUROPLUS Granulocyte Mosaic 20	Granulocytes (EOH)	---	PR3	MPO		5x5
FA 1201-####-22	EUROPLUS Granulocyte Mosaic 22	Granulocytes (EOH)	MPO	Granulocytes (HCHO)	PR3		5x5
FA 1201-####-25	EUROPLUS Granulocyte Mosaic 25	Granulocytes (EOH)	HEp-2 cells + Granulocytes (EOH)	Granulocytes (HCHO)	GBM	PR3	MPO 5x5
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FA 1202-####	IIFT: Granulocytes (MOH)	Granulocytes (MOH)					5x5