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Instruction For Use
2014-01

CE

US Market: For Research Use Only

ORG 530 ANCAcombi

NAME AND INTENDED USE

ANCAcombi is an ELISA test system for the qualitative measurement of anti-neutrophil cytoplasmic antibodies (ANCA) directed against PR3, MPO, BPI, Elastase, Cathepsin G, Lysozyme and Lactoferrin in human serum or plasma. This product is intended for professional in vitro diagnostic use only.

SYMBOLS USED ON LABELS

IVD	In vitro diagnostic medical device	MICROPLATE	Microplate
	Manufacturer	CONTROL A	Control
REF	Catalogue number	CONTROL B	Control
Σ 96	Sufficient for 96 determinations	CONTROL C	Control
LOT	Batch code		
	Use by		
2°C 8°C	Temperature limitation		
	Consult instructions for use	DILUENT	Sample Buffer P
	Keep away from sunlight	CONJUGATE	Enzyme Conjugate
	Do not reuse	TMB	TMB Substrate
	Date of manufacture	WASH	Stop solution
CE	conform to European directive 98/79/EC	STOP	Wash Buffer
		RTU	Ready to use

PRINCIPLE OF THE TEST

Purified antigens PR3, MPO, BPI, Elastase, Cathepsin G, Lysozyme and Lactoferrin are coated on to individual rows A to H of the microwell plate.
The determination is based on an indirect enzyme linked immune reaction with the following steps:
Specific antibodies in the patient sample bind to the antigen coated on the surface of the reaction wells. After incubation, a washing step removes unbound and unspecifically bound serum or plasma components. Subesquently added enzyme conjugate binds to the immobilized antibody-antigen-complexes. After incubation, a second washing step removes unbound enzyme conjugate. After addition of substrate solution the bound enzyme conjugate hydrolyses the substrate forming a blue coloured product. Addition of an acid stopps the reaction generating a yellow end-product. The intensity of the yellow color
correlates with the concentration of the antibody-antigen-complex and can be measured photometrically at 450 nm.

SUMMARY AND EXPLANATION OF THE TEST

Anti-neutrophil cytoplasmic antibodies (ANCA) represent a group of autoantibodies directed towards cytoplasmic components of the neutrophil granulocytes and monocytes. The classical methods for the determination of ANCA are immunofluorescence tests. With these indirect immunofluorescence (IF) techniques two main patterns are distinguished: a cytoplasmic (cANCA) and a perinuclear (pANCA) type.
The target antigen for 80-90 % of cANCA is proteinase 3 (PR3), a serine proteinase present in primary granules; 10-20 % of cANCA are directed to other proteins, such as bactericidal permeability-increasing protein (BPI). In rare cases, antibodies to elastase (4 %), lysozym (2 %) or cathepsin G (2 %) may show a cANCA-pattern. cANCA have also been detected in different non-rheumatic diseases. Approximately 90 % of pANCA positive sera contain autoantibodies directed to myeloperoxidase (MPO), which is located in the granules of neutrophil granulocytes. Antibodies to other antigens e.g. Lactoferrin, Elastase, Cathepsin-G and also Lysozyme often result in a similar pANCA pattern. These atypical pANCA occur in collagenosis and related inflammatory rheumatic diseases. Besides, different untypical variants of pANCA IF patterns − granulocyte specific antinuclear antibodies (GS-ANA) − are indistinguishable from pANCA. Therefore, a distinct interpretation and classification of the IF patterns is difficult and every positive IF-ANCA finding should be differentiated by ELISA techniques using the purified single antigens.

PR3 and MPO are well defined and reliable serologic markers for a definite group of primary systemic vasculitides (PSV), they are also called ANCA-associated vasculitides (AAV). The incidence is 1 in 1000 in the whole population and nearly 5 in 1000 in the elderly. The clinical appearance of AAV is characterised by manifestations in the kidneys, the lung and the respiratory tract.
PR3 is the most frequent component of cANCA and the landmark autoantigen in granulomatosis with polyangiitis (GPA, formerly named Wegener's granulomatosis) with a clinical specificity of more than 95% for the disease.
MPO, the main target antigen of pANCA, is present in 70% of patients with microscopic polyangiitis (MPA) and differentiates MPA from other autoimmune diseases.
Anti-PR3 and anti-MPO levels correlate with the clinical status; they are high in active disease. Antibody titres decrease under therapy and become undetectable after remission.
BPI (Bactericidal permeability-increasing protein) is located mainly in the primary granules of PMN but is also found on the surface of PMN and peripheral blood monocytes (PBM). BPI exhibits strong anti-microbial activity against Gram-negative bacteria and potent endotoxin-neutralizing activity, due to its high binding affinity for bacterial lipopolysaccharides. BPI is a target of ANCA in a variety of diseases of different aetiologies like cystic fibrosis, inflammatory bowel diseases (IBD), reactive arthritis, HIV, the peptide transporter complex associated with antigen presentation (TAP) deficiency and chronic obstructive pulmonary disease (COPD). Anti-BPI antibodies are detectable in Crohn's disease (23 %), ulcerative colitis (37 %) and primary sclerosing cholangitis (36 %). They constitute important markers for these diseases, but not for the ANCA-associated vasculitides.
Elastase is a serine protease with sequence homology of 54 % to proteinase 3. It mainly occurs in polymorph nuclear neutrophil granulocytes (PMN), in macrophages and endothelial cells. Degradation of proteoglycans by neutrophils is mainly due to the proteolytic activity of elastase. Moreover, Elastase is significantly involved in tissue destruction related to emphysema and rheumatoid arthritis. Autoantibodies against elastase are generally associated with inflammatory rheumatic disorders, e.g. rheumatoid arthritis, systemic lupus erythematosus or vasculitis.
Lysozyme is a glycosidase, which cleaves the glycosidic bond between C-1 of MNAc and C-4 of GlcNAc. Lysozyme is localised in the azurophilic as well as in the specific granules of neutrophils and in extracellular liquid compartments like tears and salivary, where it spreads out its antimicrobial activities against invading bacteria. Lysozyme belongs also to the pANCA group. Autoantibodies against Lysozyme occur in higher frequency in rheumatoid vasculitis and inflammatory bowel disease like ulcerative colitis.
Lactoferrin is an iron-binding protein with physiological anti microbial effect and it is a non-specific antiphlogistic defence factor at mucosal surfaces. Lactoferrin occurs in secretions such as milk, tears and saliva. Lactoferrin also resides in the specific granules of polymorph nuclear neutrophil leukocytes (PMN). During active inflammatory disease, raised serum levels of Lactoferrin can be measured.
Autoantibodies against Lactoferrin belong to the pANCA group. They occur in higher frequency in patients with rheumatoid vasculitis (RV), ulcerative colitis (CU) and primary sclerosing cholangitis (PSC).
A survey of documented clinical indications, the corresponding immunofluorescence patterns and target antigens is given in the following table:

Diseases	IF patterns	Target antigen
Systemic Vasculitic Syndromes		
Wegener´s Granulomatosis	c-ANCA, rare p-ANCA	PR3, rare MPO
Microscopic Polyangiitis	c-ANCA, p-ANCA	PR3, MPO
Churg-Strauss-Syndrome	p-ANCA	MPO
Polyarteritis nodosa	rare ANCA	rare PR3 and MPO
Unclassified Vasculitis	Rare	no PR3 and MPO
Collagen Diseases and other Rheumatic Disorders		
Rheumatoid arthritis	GS-ANA, p-ANCA, atypical ANCA	unknown, ANA, rare MPO, Lactoferrin
SLE	p-ANCA	rare MPO, Lactoferrin
Other Diseases		
Ulcerative Colitis		Cathepsin-G, Lactoferrin

CONTENTS OF THE KIT

ORG 530	Σ 96	Sufficient for 96 determinations
MICROPLATE	1	One divisible microplate consisting of 12 modules of 8 wells each. Ready to use. Product code on module: ***ANC***
CONTROL A	1x 1.5 ml	Control A (negative), containing ANCA antibodies in a serum/buffer matrix (PBS, BSA, detergent, NaN3 0.09%), yellow. Ready to use.
CONTROL B	1x 1.5 ml	Control B (cut-off), containing ANCA antibodies in a serum/buffer matrix (PBS, BSA, detergent, NaN3 0.09%), yellow. Ready to use.
CONTROL C	1x 1.5 ml	Control C (positive), containing ANCA antibodies in a serum/buffer matrix (PBS, BSA, detergent, NaN3 0.09%), yellow. Ready to use.
DILUENT	20 ml	Sample Buffer P, containing PBS, BSA, detergent, preservative sodium azide 0.09%, yellow, concentrate (5 x).
CONJUGATE	15 ml	Enzyme Conjugate containing anti-human IgG antibodies, HRP labelled; PBS, BSA, detergent, preservative PROCLIN 0.05%, light red. Ready to use.
TMB	15 ml	TMB Substrate; containing 3,3', 5,5'- Tetramethylbenzidin, colorless. Ready to use.
WASH	20 ml	Wash Buffer, containing Tris, detergent, preservative sodium azide 0.09%; 50 x conc.
STOP	15 ml	Stop solution; contains acid. Ready to use.
	1	Instruction for Use: ELISA Mini-DVD
	1	Certificate of Analysis

MATERIALS REQUIRED

- Microplate reader capable of endpoint measurements at 450 nm; optional: reference filter at 620 nm
- Data reduction software
- Multi-channel dispenser or repeatable pipette for 100 µl
- Vortex mixer
- Pipettes for 10 µl, 100 µl and 1000 µl
- Laboratory timing device
- Distilled or deionised water
- Measuring cylinder for 1000 ml and 100 ml
- Plastic container for storage of the wash solution

This ELISA assay is suitable for use on open automated ELISA processors. Each assay has to be validated on the respective automated system. Detailed information is provided upon request.

SPECIMEN COLLECTION, STORAGE AND HANDLING

- Collect whole blood specimens using acceptable medical techniques to avoid hemolysis.
- Allow blood to clot and separate the serum or plasma by centrifugation.
- Test serum should be clear and non-hemolyzed. Contamination by hemolysis or lipemia should be avoided, but does not interfere with this assay.
- Specimens may be refrigerated at 2-8°C for up to five days or stored at -20°C up to six months.
- Avoid repetitive freezing and thawing of serum or plasma samples. This may result in variable loss of antibody activity.
- Testing of heat-inactivated sera is not recommended.

STORAGE AND STABILITY

- Store test kit at 2-8°C in the dark.
- Do not expose reagents to heat, sun, or strong light during storage and usage.
- Store microplate sealed and dessicated in the clip bag provided.
- Shelf life of the unopended test kit is 18 months from day of production. Unopened reagents are stable until expiration of the kit. See labels for individual batch.
- Diluted Wash Buffer and Sample Buffer are stable for at least 30 days when stored at 2-8°C. We recommend consumption on the same day.

PROCEDURAL NOTES

- Do not use kit components beyond their expiration dates.
- Do not interchange kit components from different lots and products.

- All materials must be at room temperature (20-28°C) prior to use.
- Prepare all reagents and samples. Once started, performe the test without interruption.
- Double determinations may be done. By this means pipetting errors may become obvious.
- Perform the assay steps only in the order indicated.
- Always use fresh sample dilutions.
- Pipette all reagents and samples into the bottom of the wells.
- To avoid carryover or contamination, change the pipette tip between samples and different kit controls.
- Wash microwells thoroughly and remove the last droplets of wash buffer.
- All incubation steps must be accurately timed.
- Do not re-use microplate wells.

WARNINGS AND PRECAUTIONS

- All reagents of this kit are intended for professional in vitro diagnostic use only.
- Components containing human serum were tested and found negative for HBsAg, HCV, HIV1 and HIV2 by FDA approved methods. No test can guarantee the absence of HBsAg, HCV, HIV1 or HIV2, and so all human serum based reagents in this kit must be handled as though capable of transmitting infection.
- Bovine serum albumin (BSA) used in components has been tested for BSE and found negative.
- Avoid contact with the substrate TMB (3,3´,5,5´-Tetramethyl-benzidine).
- Stop solution contains acid, classifiaction is non-hazardous. Avoid contact with skin.
- Control, sample buffer and wash buffer contain sodium azide 0.09% as preservative. This concentration is classified as non-hazardous.
- Enzyme conjugate contains ProClin 300 0.05% as preservative. This concentration is classified as non-hazardous.

During handling of all reagents, controls and serum samples observe the existing regulations for laboratory safety regulations and good laboratory practice:

- First aid measures: In case of skin contact, immediately wash thoroughly with water and soap. Remove contaminated clothing and shoes and wash before reuse. If system fluid comes into contact with skin, wash thoroughly with water. After contact with the eyes carefully rinse the opened eye with running water for at least 10 minutes. Get medical attention if necessary.
- Personal precautions, protective equipment and emergency procedures:

Observe laboratory safety regulations. Avoid contact with skin and eyes. Do not swallow. Do not pipette by mouth. Do not eat, drink, smoke or apply makeup in areas where specimens or kit reagents are handled. When spilled, absorb with an inert material and put the spilled material in an appropriate waste disposal.

- Exposure controls / personal protection: Wear protective gloves of nitril rubber or natural latex. Wear protective glasses. Used according to intended use no dangerous reactions known.
- Conditions to avoid: Since substrate solution is light-sensitive. Store in the dark.
- For disposal of laboratory waste the national or regional legislation has to be observed.

Observe the guidelines for performing quality control in medical laboratories by assaying control sera.

PREPARATION OF REAGENTS

WASH

Dilute the contents of one vial of the buffered wash solution concentrate (50x) with distilled or deionised water to a final volume of 1000 ml prior to use.

DILUENT

Sample Buffer P: Prior to use dilute the contents (20 ml) of one vial of sample buffer 5x concentrate with distilled or deionised water to a final volume of 100 ml.

Preparation of samples

Dilute patient samples 1:100 before the assay: Put 990 µl of prediluted sample buffer in a polystyrene tube and add 10 µl of sample. Mix well. Note: Calibrators / Controls are ready to use and need not be diluted.

TEST PROCEDURE

Prepare enough microplate modules for all calibrators / controls and patient samples.

1. Pipette **100 µl** of calibrators, controls and prediluted patient samples into the wells. Incubate for **30 minutes** at room temperature (20-28 °C).

Discard the contents of the microwells and **wash 3 times** with **300 µl** of wash solution.

2. Dispense **100 µl** of enzyme conjugate into each well.
   Incubate for **15 minutes** at room temperature.
   Discard the contents of the microwells and **wash 3 times** with **300 µl** of wash solution.
3. Dispense **100 µl** of TMB substrate solution into each well.
   Incubate for **15 minutes** at room temperature
4. **Add 100 µl** of stop solution to each well of the modules
   Incubate for **5 minutes** at room temperature.
   Read the optical density at 450 nm (reference 600-690nm) and calculate the results.
   The developed colour is stable for at least 30 minutes. Read during this time.

Example for a pipetting scheme:

	1	2	3	4	5	6	7	8	9	10	11	12	Antigens coated in rows:
A	A	B	C										Reference
B	P1	P2	P3										PR3
C	P1	P2	P3										MPO
D	P1	P2	P3										BPI
E	P1	P2	P3										Elastase
F	P1	P2	P3										Cathepsin G
G	P1	P2	P3										Lysozym
H	P1	P2	P3										Lactoferrin

P1, ... patient sample A-C controls

VALIDATION

Test results are valid if the optical densities at 450 nm for calibrators / controls and the results for controls comply with the reference ranges indicated on the Certificate of Analysis enclosed in each test kit.
If these quality control criteria are not met the assay run is invalid and should be repeated.

CALCULATION OF RESULTS

First for each antigen-coated row optical density (OD) of a sample is adjusted by a lot-specific factor stated in the certificate of analysis:

OD sample adj. = OD sample * lot-specific factor

For each antigen-coated row the OD sample adj. is expressed as Index value

Index = OD sample adj. / OD Control B

PERFORMANCE CHARACTERISTICS

Calibration

This assay system is calibrated in relative arbitrary units, since no international reference preparation is available for this assay.

Measuring range

not applicable

Expected values

In a normal range study with samples from healthy blood donors the following ranges have been established with this ELISA assay: Cut-off Index 1.0

Interpretation of results

Negative: Index < 1.0
Positive: Index ≥ 1.0

Linearity

Patient samples containing high levels of specific antibody were serially diluted in sample buffer. Activity for each dilution step was calculated as Index-Value.

Sample	Dilution	Observed Index	Expected Index	O/E [%]
PR3	1:100	3.7	3.7	100
.	1:200	1.9	1.8	103
.	1:400	1.0	0.9	103
MPO	1:100	3.4	3.4	100
.	1:200	1.8	1.7	106
.	1:400	0.9	0.9	100
BPI	1:100	3.8	3.8	100
.	1:200	2.0	1.9	104
.	1:400	0.9	0.9	100
Elastase	1:100	3.0	3.0	100
.	1:200	1.6	1.5	105
.	1:400	0.7	0.7	100
Cathepsin G	1:100	2.8	2.8	100
.	1:200	1.3	1.4	91
.	1:400	0.6	0.7	83
Lysozyme	1:100	3.1	3.1	100
.	1:200	1.5	1.6	94
.	1:400	0.7	0.8	91
Lactoferrin	1:100	3.6	3.6	100
.	1:200	1.7	1.8	94
.	1:400	0.8	0.9	89

Limit of detection

not applicable

Reproducibility

Intra-assay precision: Coefficient of variation (CV) was calculated for each of three samples from the results of 24 determinations in a single run. Results for precision-within-assay are shown in the table below.
Inter-assay precision: Coefficient of variation (CV) was calculated for each of three samples from the results of 6 determinations in 5 different runs. Results for run-to-run precision are shown in the table below.

Intra-Assay		
Sample	Mean Index	CV %
PR3	1.8	3.2
MPO	2.0	2.8
BPI	1.3	4.2
Elastase	1.3	4.6
Cathepsin G	1.4	5.1
Lysozyme	1.1	4.5
Lactoferrin	1.9	3.0

Inter-Assay		
Sample	Mean Index	CV %
PR3	1.7	2.8
MPO	1.9	2.0
BPI	1.3	2.8
Elastase	1.2	3.3
Cathepsin G	1.4	2.8
Lysozyme	1.0	3.7
Lactoferrin	1.8	2.4

Interfering substances

No interference has been observed with haemolytic (up to 1000 mg/dl) or lipemic (up to 3 g/dl triglycerides) sera or plasma, or bilirubin (up to 40 mg/dl) containing sera or plasma. Nor have any interfering effects been observed with the use of anticoagulants (Citrate, EDTA, Heparine). However for practical reasons it is recommended that grossly hemolyzed or lipemic samples should be avoided.

Study results

Sample Description	n	n Pos	%
ANCA vasculitis	102	92	90.2
/ non-ANCA vasculitis			
or other conditions			
healthy controls	234	32	13.7
or non-rheumatological			
or non-ANCA vasculitis			

		Immunological Pos	Immunological Neg	
ORG 530	Pos	92	32	
	Neg	10	202	
		102	234	336
Sensitivity:	90.2	%		
Specificity:	86.3	%		
Overall agreement:	87.5	%		

LIMITATIONS OF THE PROCEDURE

This assay is a diagnostic aid. A definite clinical diagnosis should not be based on the results of a single test, but should be made by the physician after all clinical and laboratory findings have been evaluated concerning the entire clinical picture of the patient. Also every decision for therapy should be taken individually.
The above pathological and normal reference ranges for antibodies in patient samples should be regarded as recommendations only. Each laboratory should establishe its own ranges according to ISO 15189 or other applicable laboratory guidelines.

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1. **100 µl** Standards, Kontrollen und verdünnte Patientenproben pipettieren
   → **30 Minuten** bei Raumtemperatur inkubieren
   → Inhalt der Platte verwerfen und 3 mal mit **300 µl** Waschpuffer waschen
2. **100 µl** Enzymkonjugatlösung pipettieren
   → **15 Minuten** bei Raumtemperatur inkubieren
   → Inhalt der Platte verwerfen und 3 mal mit **300 µl** Waschpuffer waschen
3. **100 µl** Substratlösung pipettieren
   → **15 Minuten** bei Raumtemperatur inkubieren
4. **100 µl** Stopplösung zugeben
   → Platte **5 Minuten** stehenlassen
   → Bei **450 nm** messen

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