

Revised 31 May 2007

**PRINCIPLE OF THE PROCEDURE**

ANCA combi is an indirect solid phase enzyme immunometric assay (ELISA). It is designed for the qualitative determination of anti-neutrophil cytoplasmic antibodies (ANCA) directed against PR3, MPO, BPI, Elastase, Cathepsin G, Lysozyme and Lactoferrin. The assay system recognizes IgG class autoantibodies. The wells of the microplate were coated with mixed antigen, PR3, MPO, BPI, Elastase, Cathepsin G, Lysozyme and Lactoferrin in row A to H, respectively. The microplate can be divided into 12 modules of 8 wells each or can be used completely for 96 determinations. During this procedure the binding of present autoantibodies, as well as the formation of the sandwich complexes and enzymatic color reaction take place during three different reaction phases:

**Phase 1:**

Controls and prediluted patient samples are pipetted into the wells of the microplate. Any present antibodies bind to the immobilized antigens. After 30 minutes incubation the microplate is washed with wash buffer for removing non-reactive serum components.

**Phase 2:**

An anti-human-IgG horseradish peroxidase conjugate solution is pipetted into the wells of the microplate to recognize the autoantibodies bound to the immobilized antigens. After 15 minutes incubation any excess enzyme conjugate, which is not specifically bound is washed away with wash buffer.

**Phase 3:**

A chromogenic substrate solution containing TMB (3,3',5,5'-Tetramethyl-benzidine) is dispensed into the wells. During 15 minutes of incubation the color of the solutions change into blue. Adding 1 M hydrochloric acid as stop solution stops color development. The solutions color change into yellow. The amount of colour is directly proportional to the concentration of IgG present in the original sample. The optical density of the controls serves as reference for the determination of the cut-off OD. Patient results may be classified in terms of negative or positive. To read the optical density a microplate reader with a 450 nm filter is required. Bi-chromatic measurement with a 600-690 nm reference is recommended.

**CLINICAL RELEVANCE**

The acronym ANCA (Antineutrophil Cytoplasmic Autoantibodies) is defined by an accumulation of autoantibodies with specificity against different granulocytic, monozytic and probably endothelial cytoplasmic antigens. Autoantibodies against endocellular components of neutrophile granulocyte (PMN) are known for a longer period. At first, in 1964 they have been described as granulocyte specific factor directed against anti nuclear components (GS-ANA). Under the term ANCA, they were first reported in 1982 in a few patients who had necrotizing glomerulonephritis without immune deposits, but the relevance was still unclear. The next report of ANCA was in 1984 where these autoantibodies has been observed in four patients with systemic vasculitis and three of whom had also necrotizing glomerulonephritis. In 1985, a collaborative study in the Netherlands and Denmark described these factors, showing a high sensitivity for active Wegener's granulomatosis, as APCA (Anti Cytoplasmic Antibodies) and they suggested that APCA could be used as a marker for disease activity. In 1988 further investigations substantiated the association of ANCA with Wegener's Granulomatosis and polyarteritis nodosa, and also noted that ANCA occur in patients with pauciimmune ("idiopathic") necrotizing and crescentic glomerulonephritis who have no evidence for extrarenal disease. Proteinase 3 (PR3), showing a central accentuated pattern (cANCA) has been described as major antigen for Wegener's Granulomatosis. A new pattern, showing a high specificity for the microscopic Polyangiitis, with perinuclear fluorescence (pANCA) was also observed in 1988. At first, only Myeloperoxidase (MPO) was described as antigen. In the last years, newer investigations discovered

Revised 31 May 2007

and characterized a couple of new pANCA antigens: Elastase, Cathepsin G, Lysozyme and Lactoferrin. In the meantime, PR3 and MPO are well defined as reliable serological markers for a definite group of primary systemic vasculitides (PSV), which were also named ANCA associated vasculitides (AAV). The occurrence of AAV is clearly higher than supposed. The incidence is 1.5 per 1000 and in the group of older persons nearly 5 per 1000. The clinical appearance of the AAV is characterized through manifestations in lung, kidney and respiratory tract. Up to now, ANCA screening has been done with immunofluorescence techniques, but often there have been difficulties in the evaluation and in clinical findings. Therefore, the results have to be scrutinized with counter examinations on other cells or in other test systems like ELISA. Moreover it was not possible to differentiate the single cANCA and pANCA antigens.

### Proteinase 3:

The major antigen for the cANCA reactivity is the neutral serin protease 3 (synonyms: p29, AGP7, Wegener autoantigen), which belongs to the Trypsin/Chymotrypsin family. In 1988 several groups showed that the antigen is a protein with a molecular weight of 29 kDa. Ohlsson and Olsson already described PR3 in 1973 under the name neutrophil collagenase. In the meantime it seems certain, that autoantibodies against PR3 are highly specific as serological marker for the diagnosis of Wegener's granulomatosis (specificity: initial phase 50%, generalization phase > 90%). Moreover there is a correlation between the concentration of the autoantibodies and the disease activity.

### Myeloperoxidase:

Agner first isolated Myeloperoxidase in 1941, which gave it the name "verdoperoxidase", because of its green color and ability to catalyze peroxidase reactions. It is MPO that gives pus its greenish tone. In nearly 60% of the pANCA findings MPO is the major antigen. The occurrence of autoantibodies against MPO is classified as relevant marker for the rapid progressive nephritis. Moreover these antibodies occur in 70-90% in all patients with serious kidney injury. Over and about they have also been detected at the Churg-Strauß-Syndrom (CSS), Microscopic Polyangiitis (MPA) and other vasculitis diseases. The concentration of the autoantibodies correlates well with the disease activity of MPA. MPA is also characterized by clinical manifestations of lung, kidney and respiratory tract, but these manifestations are, in contrast to WG, not granulomatous. However, these antibodies have, in contrast to the high specificity of PR3 antibodies for WG, a minor specificity of 60% in the diagnosis of MPA. The absence of autoantibodies against MPO and PR3, by simultaneous detection of ANA can be used as a tool for differential diagnosis between AAV and SLE induced vasculitis.

### BPI:

Bactericidal permeability-increasing protein, BPI is a membrane-located protein of 55kDa molecular weight and is classed as an ANCA-antigen of polymorph-nuclear granulocytes and monocytes that bind endotoxin. Its autoantibodies are now classified as cANCA. Due to BPIs high affinity to lipopolysaccharides its anti-microbial effect against Gram-negative bacteria is significant. BPI is splitted and thus inactivated by using elastase or other serine protease. Autoantibodies against BPI are above all detected in chronically infectious intestinal diseases such as Morbus Crohn or colitis ulcerosa. In contrast to anti-MPO and anti-PR3 autoantibodies, those against BPI seem not to have any association with vasculitis.

### Elastase:

Elastase is a serine protease with a sequence homology of 54% to that of proteinase 3. It occurs mainly in polymorph-nuclear neutrophilic granulocytes (PMN), in macrophages and endothelial cells. The dismantling of proteoglycans by neutrophils is mainly due to elastase' proteolytic activity. Furthermore, elastase participates decisively in tissue destruction connected with emphysemas and rheumatoid arthritis. Autoantibodies against this antigen are generally associated with inflammatory rheumatic disorders, e.g. rheumatoid arthritis and vasculitis.

### Cathepsin G:

Revised 31 May 2007

The cathepsins belong to a group of intracellular proteases mainly found in lysosomes, especially of the spleen, the liver and the kidney. Cathepsin G is a serine protease and a further pANCA antigen. It participates to a great part in the destruction of osteoid tissue as of its hydrolytic properties. The autoantibodies against Cathepsin G occur mainly in collagenosis and other related inflammatory rheumatic diseases, e.g. SLE, Sjögren syndrome and Felty syndrome.

#### Lysozyme:

In 1922, Lysozyme (LZ) was accidentally discovered by Alexander Flemming. LZ is a glycosidase consisting of 129 amino acid residues with a molecular weight of 14,6 kDa, which decomposes the glycosidic bond between C-1 of MNAC and C-4 of GlcNAc. Lysozyme is localized 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. LZ belongs also to the pANCA and autoantibodies against Lysozyme occur in higher frequency in rheumatoid vasculitis and inflammatory bowel disease like colitis ulcerosa.

#### Lactoferrin:

Lactoferrin (LF) is an iron-binding protein, with a molecular weight of 77-93 kDa, which occurs in high concentrations in secretions at mucosa surfaces, in tears and in milk. LF also resides in the specific granules of polymorphonuclear neutrophil leukocytes (PMN) and becomes exocytosed upon PMN activation. During active inflammatory disease, raised serum levels of LF can be measured. The physiological anti microbial effect of Lactoferrin depends on its iron-binding capacity, because most of the bacteria require iron for their own physiological pathways. LF inhibits myelopoiesis, prevents complement activation and prevents the formation of hydroxyl radicals. It is quite possible that LF has several important roles, like secretory IgA, as a non-specific antiphlogistic defense factor at mucosal surfaces. LF belongs to the pANCA, depending on the redistribution from the granules toward the nuclei, upon ethanol fixation. Autoantibodies against Lactoferrin occur in higher frequency in patients with rheumatoid vasculitis (RV), colitis ulcerosa (CU) and primary sclerosing cholangitis (PSC).

#### SPECIFICITY

The microplate is coated with the antigens PR3, MPO, BPI, Elastase, Cathepsin G, Lysozyme and Lactoferrin, respectively. All antigen preparations are highly purified by affinity chromatography. The ANCA combi test is specific only for autoantibodies directed to these antigens. No crossreactivities have been observed.

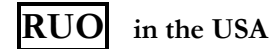
#### CALIBRATION

Since no international reference preparations for ANCA autoantibodies are available, the assay system is calibrated arbitrarily.

#### WARNINGS AND PRECAUTIONS

All reagents of this test kit are strictly intended for in vitro use only. In the United States, this kit is intended for Research Use Only. Please adhere strictly to the sequence of pipetting steps provided in this protocol. Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. All reagents should be stored refrigerated at 2 - 8 °C in their original container. Do not interchange kit components from different lots. The expiration dates stated on the labels of the shipping container and all vials have to be observed. Do not use kit components beyond their expiration dates. Allow all kit components and specimen to reach room temperature prior to use and mix well. During handling of all kit reagents, controls and serum samples observe the existing legal regulations. The following precautions should be taken handling potentially infectious materials:

- Do not eat, drink or smoke in areas where specimens or kit reagents are handled

**Revised 31 May 2007**

- Do not pipette by mouth  
 - Wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.  
 The test kit contains components of human origin which, when tested by FDA-licensed methods, were found negative for hepatitis B surface antigen and for HIV antibody. No known test can guarantee, however, that products derived from human blood will not be infectious. Handle, therefore, all reagents and human blood derivatives, like plasma or serum samples, as if capable of transmitting infection. Avoid contact with the TMB (3,3',5,5'-Tetramethyl-benzidine). If TMB comes into contact with skin wash thoroughly with water and soap. The stop solution contains hydrochloric acid. If it comes into contact with skin, wash thoroughly with water and seek medical attention. Avoid contact between the buffered Peroxide Solution and easily oxidized materials; extreme temperatures may initiate spontaneous combustion.

**MATERIALS SUPPLIED**

<b>Package size</b>	<b>96 determ.</b>
Divisible microplate consisting of 12 modules of 8 wells, Coated with highly purified antigens: PR3, MPO, BPI, Elastase, Cathepsin G, Lysozyme and Lactoferrin ANCA Controls in a PBS/BSA matrix	1  3 vials, 1.5 ml each
Negative Control (A)	
Cut-off Control (B)	
Positive Control (C)	
Sample buffer, yellow, Concentrate	1 vial, 20 ml
Enzyme conjugate solution, (light red) containing polyclonal .	1 vial, 15 ml
Rabbit anti-h-IgG-IgG; labelled with horseradish peroxidase	
TMB substrate solution	1 vial, 15 ml
Stop solution (1 M hydrochloric acid)	1 vial, 15 ml
Buffered wash solution, Concentrate	1 vial, 20 ml

**CONTROLS**

A set of three controls is provided with the kit.

**TECHNICAL DATA**

Sample material:	serum or plasma
Required sample volume:	10 µl of sample to be diluted 1:100 with sample buffer 100 µl prediluted sample per single determination
Total incubation time:	60 minutes at room temperature (20 - 28 °C)
Storage:	refrigerated at 2 - 8 °C
Shelf life:	12 months after manufacturing or until the expiration date printed on the labels
Package size:	96 tests

**MATERIALS REQUIRED****Equipment**

- Microplate reader capable for endpoint measurements at 450 nm
- Vortex mixer
- Pipets for 10 µl, 100 µl and 1000 µl

Revised 31 May 2007

**Preparation of reagents**

- Distilled water
- Graduated cylinder for 100 and 1000 ml
- Plastic container for storage of the wash solution

**Optional**

- Multi-Chanel Dispenser or repeatable pipet for 100  $\mu$ l
- Data reduction software

**SPECIMEN COLLECTION AND PREPARATION**

For determination of ANCA antibodies serum or plasma are the preferred sample matrixes. All serum and plasma samples are prediluted 1: 100 with sample buffer. Therefore 10  $\mu$ l of sample may be diluted with 1000  $\mu$ l of sample buffer. The patients need not to be fasting, and no special preparations are necessary. Collect blood by venipuncture into vacutainers and separate serum or plasma from the cells by centrifugation after clot formation. Samples may be stored refrigerated at 2 - 8 °C for at least 5 days. For longer storage of up to six months samples should be stored frozen at -20 °C. To avoid repeated thawing and freezing the samples should be aliquoted. Neither Bilirubin nor Hemolysis has significant effect on the procedure.

**PREPARATION AND STORAGE OF REAGENTS**

All components of this test kit are supplied in a liquid format and ready to use, except the sample buffer and wash buffer. When stored refrigerated at 2 - 8 °C the components are stable for at least 30 days after opening or until the expiration date printed on the labels. Remaining modules of the microplate should be stored refrigerated at 2 - 8 °C protected from moisture; store together with desiccant and carefully sealed in the plastic bag.

**Preparation of sample buffer**

Dilute the contents of each vial of the sample buffer concentrate (5x) with distilled water to a final volume of 100 ml prior to use. Store refrigerated: stable at 2 - 8 °C for at least 30 days after preparation or until the expiration date printed on the label.

**Preparation of buffered wash solution**

Dilute the contents of each vial of the buffered wash solution concentrate (50x) with distilled water to a final volume of 1000 ml prior to use. Store refrigerated: stable at 2 - 8 °C for at least 30 days after preparation or until the expiration date printed on the label.

**NOTES ON TECHNIQUE**

Control sera or pools should routinely be assayed as unknowns to check performance of the reagents and the assay. For all controls, the respective concentrations are provided on the labels of each vial. Using these concentrations a calibration curve may be calculated to read off the patient results semi-quantitatively.

**Pipetting and Sample Handling**

Use a disposable-tip micropipette to dispense sera and plasma samples. Pipet directly to the bottom of the wells. To avoid carryover contamination changes the tip between samples. Patient samples expected to contain high concentrations should be additionally diluted with sample buffer before. Additional dilutions must be considered during calculation.

Revised 31 May 2007

**IMMUNOASSAY PROCEDURE**

Do not interchange components of different lots. All components should be at room temperature before use. Dilute all patient samples 1:100 with sample buffer before assay. Therefore combine 10 µl of sample with 1000 µl of sample buffer in a polystyrene tube. Mix well. Calibrators and controls are ready to use and need not to be diluted.

1. Prepare a sufficient number of microplate modules to accommodate controls and prediluted patient samples in duplicates.

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>
<b>Reference antigen a</b>	CA	CB	CC			
<b>PR3 b</b>	P1	P2	P3	P...		
<b>MPO c</b>	P1	P2	P3	P..		CA -CC Controls A to C
<b>BPI d</b>	P1	P2	P3	P..		P1, P2,.. Patient sample
<b>Elastase e</b>	P1	P2	P3			
<b>Cathepsin G f</b>	P1	P2	P3			
<b>Lysosyme g</b>	P1	P2	P3			
<b>Lactoferrin h</b>	P1	P2	P3			

- Pipet 100 µl of 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.
- Dispense 100 µl of enzyme conjugate solution 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.
- Dispense 100 µl of TMB substrate solution into each well.
- Incubate for 15 minutes at room temperature protected from light.
- Add 100 µl of stop solution to each well of the modules and leave untouched for 5 minutes.
- Read the optical density at 450 nm and calculate the results. Bi-chromatic measurement with reference at 600-650 nm is recommended. The developed color is stable for at least 30 minutes. Read optical densities during this time.

**CALCULATION OF RESULTS**

Evaluation of the ANCA combi test is carried out by comparison of the optical densities of the patient sample with the optical density of the Cut-off Control. Previously, each patient`s OD must be corrected by a specific factor (please refer to the certificate).

$$\text{Index - value} = \frac{\text{OD}_{\text{sample}} \times \text{Factor}}{\text{OD}_{\text{Cut-Off control B}}}$$

For each patient sample 7 Index-values should be calculated according to the following equation:

Revised 31 May 2007

A sample is positive for one ANCA parameter if its Index-value is  $\geq 1.0$ :

**ANCA combi  
(Index-value)**

**Negative:** < 1.0  
**Positive:**  $\geq 1.0$

For detailed quantitative analyses we recommend to use the individual ANCA-ELISAs.

**CALCULATION EXAMPLE**

The chart below shows typical results for ANCA combi. These data are intended for illustration only and should not be used to calculate results from another run.

Sample No.	Anti-	Row	OD <sub>sample</sub>	Factor	OD <sub>cutoff</sub>	Index-Value	Result	Result
1	PR3	B	0.083	1.10		0.22	Negative	Neg.
1	MPO	C	0.920	1.00		2.19	Positive	Pos.
1	BPI	D	0.138	1.25		0.41	Negative	Neg.
1	Elastase	E	0.133	0.85	0.420	0.27	Negative	Neg.
1	Cath. G	F	0.505	0.75		0.90	Negative	Neg.
1	Lysozyme	G	0.420	1.00		1.00	Positive	Pos.
1	Lactoferrin	H	0.112	0.80		0.21	Negative	Neg.
2	PR3	B	2.143	1.10		5.61	High pos.	High Pos.
2	MPO	C	0.240	1.00	0.420	0.57	Negative	Neg.
2	BPI	D	0.079	1.25		0.24	Negative	Neg.
2	...	...	...	...		...	...	...

**REFERENCES**

- Hauschild, S.; Schmitt, W.H.; Csernok, E.; Flesch, B. K.; Rautmann, A.; Gross, W.L. ANCA in systemic vasculitides, collagen vascular diseases, rheumatic disorders and inflammatory bowel diseases. ANCA-associated Vasculitides: Immunological and clinical aspects. Edited by W. L. Gross, Plenum Press, New York, 1993, pp. 245 - 251.
- Schmitt, W.H.; Csernok, E.; Flesch, B. K.; Hauschild, Gross, W.L. Autoantibodies directed against Lysozyme: A new target Antigen for anti-neutrophil cytoplasmatic antibodies (ANCA). ANCA-associated Vasculitides: Immunological and clinical aspects. Edited by W. L. Gross, Plenum Press, New York, 1993, 267 - 272.
- Skogh, T. and Peen, E. Lactoferrin, anti-Lactoferrin antibodies and inflammatory disease. ANCA-associated Vasculitides: Immunological and clinical aspects. Edited by W. L. Gross, Plenum Press, New York, 1993, pp. 533 - 538.
- Stoffel, M. P.; Csernok, E.; Herzberg, C.; Johnston, T.; Carroll, S.F. and Gross, W. L. Anti-neutrophil cytoplasmatic antibodies (ANCA) directed against bactericidal/permeability increasing protein (BPI): a new seromarker for inflammatory bowel disease and associated isorders. Clin Exp. Immunol. 1996, No. 104, pp. 54 - 59.
- Zhao, M. H.; Jayne, D. R. W.; Ardiles, L. G.; Culley, F.; Hodson, M.E. and Lockwood. Autoantibodies against bactericidal/permeabilty increasing protein in patients with cystic fibrosis. J. Med. 1996, No. 89, pp. 259 - 265.

DRG



DRG® ANCA combi (EIA-3595)



**RUO** in the USA

Revised 31 May 2007

6. Wiik, A.; Stumman, L.; Kjeldsen, L.; Borregaard, N.; Ullman, S.; Jacobsen, S. and Halberg, P. The diversity of perinuclear antineutrophil cytoplasmic antibodies (pANCA) antigens. Proceedings of the 6th international ANCA workshop, France - July 1995, pp. 15 - 17.7. Jenette, J. C. and Falk, R. J..
7. Antineutrophil cytoplasmic autoantibodies and associated diseases: A review. Am. J. of Kidney Disease 1990, Vol XV, No. 6, pp. 517 - 529.
8. Schmitt, W. H. and Gross, W. L.. Antineutrophile zytoplasmatische Antikörper (ANCA). Internist 1995, No. 36, pp. 282 - 290.
9. Gross, W. L. and Rheinhold-Keller, E. ANCA-assoziierte Vaskulitiden (Wegener Granulomatose, Churg-Strauß-Syndrom, Mikroskopische Polyangiitis) - 1. Systematik, Pathogenese and Klinik. Z. Rheumatol (1995), No. 54, pp. 279 - 290.
10. de Grot, K.; Schnabel, A. and Gross, W. L. ANCA-assoziierte Vaskulitiden (Wegener Granulomatose, Churg-Strauß-Syndrom, Mikroskopische Polyangiitis) - 1. Diagnostisches Procedere. Z. Rheumatol (1995), No. 54, pp. 291 - 302.