



# INSTRUCTION MANUAL

REF 4034

June 17<sup>th</sup>, 2008

## ANCAcombi

- 12 x 7 determinations -



IVD *In vitro* diagnostic device

Enzyme immunoassay for the determination of IgG antibodies to proteinase 3, myeloperoxidase, bactericidal permeability-increasing protein, elastase, cathepsin G, lysozyme and lactoferrin in human serum and plasma

<b>REF</b>	Catalogue number	<b>LOT</b>	Batch code
	Consult accompanying documents		Manufactured by
	Temperature limitation		Use by
	Consult operating instruction		Biological risk



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### INTENDED USE

ANCAcombi is used for the quantitative or semi-quantitative determination of autoantibodies to proteinase 3 (PR3), myeloperoxidase (MPO), bactericidal permeability-increasing protein (BPI), elastase, cathepsin G, lysozyme and lactoferrin in human serum or plasma for the differential diagnosis of systemic vasculitis (SV).

Pathogenesis of SV is characterized by inflammatory processes of different blood vessel walls and resulting morphological alterations. Both, arteries and veins can be affected simultaneously. Classification of SV by the Chapel Hill Consensus Conference 1992 is based on the kind of blood vessel affected: vasculitides of large vessels (Giant cell arteritis, Takayasu-arteritis), vasculitides of medium vessels (Polyarteritis nodosa, Kawasaki-syndrome) and vasculitides of small vessels (Wegener's granulomatosis, Churg-Strauss syndrome, microscopic polyangiitis, Purpura Schönlein-Henoch).

The clinical picture is mainly characterized by general symptoms like exhaustion, fever and weight loss. During the further course of disease symptoms vary dependent on which kind of vessels are affected.

For the serological diagnosis of SV anti-neutrophil cytoplasmic antibodies (ANCA) play an important role. These antibodies are usually determined by indirect immunofluorescence (IIF) of ethanol-fixed human neutrophils. Depending on the pattern of the IIF cytoplasmic ANCA (cANCA) and perinuclear ANCA (pANCA) are distinguished. PR3 has been identified as the responsible autoantigen causing the IIF pattern of cANCA. PR3 autoantibodies are described to be pathognomonic for Wegener's granulomatosis.

The IIF pattern of pANCA is mainly caused by the reactivity of MPO which is a cationic protein with a molecular weight of 146 kDa found in azurophilic granules. However, other cellular components like lactoferrin, cathepsin G, lysozyme, BPI, and elastase cause pANCA patterns, too and are therefore included in the group of pANCA antigens. MPO autoantibodies occur in a variety of vasculitides such as microscopic polyangiitis, Churg-Strauss syndrome and Polyarteritis nodosa.

Jennete JC, Falk RJ, Andrassy K, Bacon PA, Churg J, Gross WL, Hagen EC, Hoffman GS, Hunder GG, Kallenberg CGM, McCluskey RT, Sinicio RA, Rees AJ, vanEs LA, Waldherr R, Wiik A: Nomenclature of systemic vasculitides: proposal of an international conference. *Arthritis Rheum* (1994) 37: 187 - 192

Lüdemann J, Utrecht B, Gross WL: Antineutrophil cytoplasm antibodies in Wegener's granulomatosis recognize an elastinophil enzyme. (1990) *J Exp Med* 171: 375 - 362

### PRINCIPLE OF THE TEST

ANCAcombi is an enzyme immunoassay for the quantitative or semi-quantitative determination of IgG antibodies to PR3, MPO, BPI, elastase, cathepsin G, lysozyme and lactoferrin.

The antibodies of standards, control and diluted patient samples react with purified human antigens immobilized on the solid phase of microtiter plates. Following an incubation period of 30 min at room temperature, unbound sample components are removed by a wash step.

The bound IgG antibodies react specifically with anti-human-IgG conjugated to horseradish peroxidase (HRP). Within the incubation period of 30 min at room temperature, excessive conjugate is separated from the solid-phase immune complexes by the following wash step.

HRP converts the colorless substrate solution of 3,3',5,5'-tetramethylbenzidine (TMB) added into a blue product. The enzyme reaction is stopped by dispensing an acidic solution into the wells after 30 min at room temperature turning the solution from blue to yellow.

The optical density (OD) of the solution at 450 nm is directly proportional to the amount of specific antibodies bound. The standard curve is established by plotting the antibody concentrations of the standards (x-axis) and their corresponding OD values (y-axis) measured. The concentration of antibodies of the specimen is directly read off the standard curve. Alternatively binding ratios are calculated by dividing the respective OD of the specimen by the OD of the cut-off control.

## PATIENT SAMPLES

### Specimen collection and storage

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. Plasma can be used, too. Lipaemic, hemolytic or contaminated samples should not be run.

Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at -20 °C.

### Preparation before use

Allow samples to reach room temperature prior to assay. Take care to agitate patient samples gently in order to ensure homogeneity.

**Note:** *Patient samples have to be diluted 1 + 100 (v/v), e.g. 10 µl sample + 1 ml sample diluent (C), prior to assay.*

The samples may be kept at 2 - 8 °C for up to three days. Long-term storage requires -20 °C.

## TEST COMPONENTS FOR 96 WELLS

<b>A</b> <b>Ag</b> <b>96</b>	<b>Microtiter plate</b> , 12 breakable strips per 8 wells coated with PR3, MPO, BPI, elastase, cathepsin G, lysozyme and lactoferrin,	1 vacuum sealed with desiccant
<b>B</b> <b>BUF</b> <b>WASH</b> <b>50x</b>	<b>Concentrated wash buffer</b> sufficient for 1000 ml solution	20 ml concentrate capped white
<b>C</b> <b>DIL</b> <b>5x</b>	<b>Concentrated sample diluent</b>	20 ml concentrate capped white
<b>D</b> <b>CONJ</b> <b>G</b>	<b>Conjugate</b> containing anti-human-IgG- (sheep) coupled with HRP	15 ml ready for use capped blue
<b>E</b> <b>SOLN</b> <b>TMB</b>	<b>Substrate</b> 3,3',5,5'-tetramethylbenzidine in citrate buffer containing hydrogen peroxide	15 ml ready for use capped black
<b>F</b> <b>HCl</b> <b>1.0 M</b>	<b>Stop solution</b> 1.0 hydrochloric acid	15 ml ready for use capped white
<b>1 - 4</b> <b>CAL</b>	<b>Standards</b> (human serum diluted) conc.: 0, 10, 30, 100 U/ml	1.5 ml each ready for use capped white
<b>Co</b> <b>CONTROL</b>	<b>Cut-off control</b> (human serum diluted)	1.5 ml ready for use capped blue

### Materials required

- micropipettes
- multi-channel pipette or multi-pipette trough for multi-channel pipette
- 8-channel wash comb with vacuum pump and waste bottle or microplate washer
- distilled or de-ionized water
- glassware

### Size and storage

ANCAcombi has been designed for the determinations of antibodies to 7 antigens in 12 samples. The first row is coated with calibration antigen for calculation of concentrations or cut-off.

The expiry date of each component is reported on its respective label that of the complete kit on the box labels.

Upon receipt, all components of the ANCAcombi have to be kept at 2 - 8 °C, preferably in the original kit box.

After opening all kit components are stable for at least 2 months, provided proper storage.

### Preparation before use

Allow all components to reach room temperature prior to use in the assay.

The microtiter plate is vacuum-sealed in a foil with desiccant. The plate consists of a frame and strips with breakable wells. Allow the sealed microplate to reach room temperature before opening. Unused wells should be stored refrigerated and protected from moisture in the original cover carefully resealed.

Prepare a sufficient amount of wash solution by diluting the concentrated wash buffer 50 times with de-ionized or distilled water. For example, dilute 1 ml of the concentrate with 49 ml of distilled water per strip. The wash solution prepared is stable at 2 - 8 °C up to 30 days.

Prepare a sufficient amount of sample diluent by diluting the concentrated diluent 5 times with de-ionized or distilled water. For example, dilute 10 ml of the concentrate with 40 ml of distilled water. The sample diluent prepared is stable at 2 - 8 °C up to 30 days.

Make sure the soak time of the wash buffer in the wells is at least 5 seconds per wash cycle.

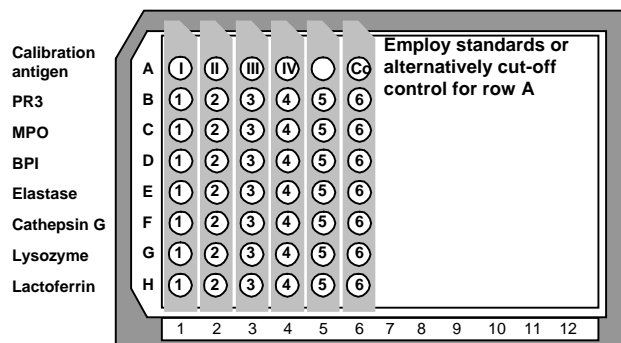
Avoid exposure of the TMB substrate solution to light!

## ASSAY PROCEDURE

- Dilute patient sera with sample diluent (C) 1 + 100 (v/v), e.g. 10 µl sample + 1 ml sample diluent (C)
- Avoid any time shift during pipetting of reagents and samples.

1. Bring all reagents to room temperature (18-25°C) before use. Mix gently without causing foam.
2. Dispense **100 µl** standards (1-4) or **100 µl** cut-off control (Co) into row A and **100 µl** diluted patient samples into the respective wells (see pipetting format).
3. Seal plate, incubate **30 min** at room temperature (18-25°)
4. Decant, then wash each well **three times** using **300 µl** wash solution (made of B).
5. Add **100 µl** of conjugate (D) solution to each well.
6. Seal plate, incubate **30 min** at room temperature (18-25°)
7. Decant, then wash each well **three times** using **300 µl** wash solution (made of B).
8. Add **100 µl** of substrate (E) to each well.
9. Incubate **30 min** protected from light at room temperature (18-25°).
10. Add **100 µl** of stop solution (F) to each well and mix gently.
11. Read the OD at **450 nm** versus 620 or 690 nm within **30 min** after adding the stop solution.

## Pipetting Format



### DATA PROCESSING

ANCAcombi allows both quantitative and semi - quantitative evaluation of results.

#### Semi - quantitative evaluation

Results are interpreted by calculating the binding index (BI) using the cut-off control.

$$BI = OD_{\text{sample}} / OD_{\text{cut-off control}}$$

This calculation can be done by the integrated evaluation software of the microplate reader used, too.

#### Quantitative evaluation

We recommend log / lin processing for best results.

The standard curve is established by plotting the mean OD-values of the standards 1 - 4 on the ordinate, y-axis, (lin. scale) versus their respective IgG concentrations on the abscissa, x-axis, (log. scale).

ANCA antibody concentrations of the unknown samples are directly read off in U/ml against the respective OD values.

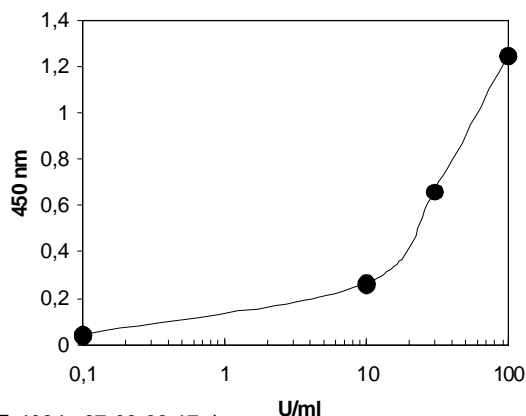
ANCAcombi may be used also with Computer Assisted Analysis using software able to plot log/lin curves with four-parameter fit.

Using the recommended dilution of 1 + 100 (v/v) for patient's sera, no correction factor is necessary, as all other components of the kit are supplied accordingly.

#### Example of Typical Assay Results

well	OD (a)	OD (b)	OD (mean)	U/ml
Standard 1	0.043	0.040	0.042	0
Standard 2	0.269	0.261	0.265	10
Standard 3	0.657	0.669	0.663	30
Standard 4	1.214	1.290	1.252	100
Patient 1	0.341	0.361	0.351	15

#### TYPICAL STANDARD CURVE



Specimens with an OD > standards four, should be diluted with ANCA negative serum and tested again. Results are multiplied with the dilution factor chosen.

#### Test validity

The test run is valid if:

- the mean OD of the standard 1 is  $\leq 0.15$
- the mean OD of the standard 4 is  $\geq 0.8$

If the above mentioned quality criteria are not met, repeat the test and make sure that the test procedure is followed correctly (incubation times and temperatures, sample and wash buffer dilution, wash steps etc.). In case of repeated failure of the quality criteria contact your supplier.

### REFERENCE VALUES

	U/ml	BI
negative	< 12	0.8
equivocal	12 - 18	0.8 - 1.2
positive	> 18	1.2

It is recommended that each laboratory establishes its own normal and pathological reference ranges for serum ANCA levels, as usually done for other diagnostic parameters, too. Therefore, the above mentioned reference values provide a guide only to values which might be expected.

#### Limitations of Method

Healthy individuals should be tested negative by ANCAcombi. However, ANCA autoantibody positive apparently healthy persons do occur.

Any clinical diagnosis should not be based on the results of in vitro diagnostic methods alone. Physicians are supposed to consider all clinical and laboratory findings possible to state a diagnosis.

### PERFORMANCE CHARACTERISTICS

#### Calibration

No international reference material for these parameters are available so the assay is calibrated in arbitrary units.

#### Linearity

Positive selected serum samples have been tested by this assay and found to dilute linearly. However, due to the heterogeneous nature of human autoantibodies there might be sera that do not follow this rule.

#### Sensitivity

The analytical sensitivity of this assay has been determined at 1.0 U/ml.

#### Specificity

No cross reactivity to other autoantigens but stated under test components have been found.

#### Precision

sample	Intraassay		Interassay	
	mean U/ml	CV %	mean U/ml	CV %
PR3	136	3,5	128	3,5
MPO	72,6	3,7	70,9	3,7
BPI	33,4	4,8	35,3	4,8
Elastase	24,9	5,7	22,8	5,7
Cathepsin-G	105	3,4	110	3,4
Lysozyme	36,9	4,1	32,4	4,7
Lactoferrin	86,4	4,1	84,6	5,8

## INCUBATION SCHEME

# ANCAcombi (4034)

<b>Dilute patients sample</b>	<b>10 µl sample + 1 ml sample diluent (made from C)</b>
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1	Bring all ready for use reagents to room temperature (18-25°C) before use.				
2	Pipette	Standards (1 - 4) or Cut-off control (Co) 1 + 100 prediluted sera	100 µl	100 µl	100 µl
3	Incubate 30 minutes , room temperature (18-25°C)				
4	Wash Decant, Dispense 3 x 300 µl (made of B)				
5	Pipette conjugate (D)		100 µl	100 µl	100 µl
6	Incubate 30 minutes, room temperature (18-25°C)				
7	Wash Decant, Dispense 3 x 300 µl (made of B)				
8	Pipette substrate (E)		100 µl	100 µl	100 µl
9	Incubate protected from light 30 minutes, room temperature (18-25°C)				
10	Pipette stop solution (F)		100 µl	100 µl	100 µl
11	Measure 450 nm versus 620 (690) nm				

### SAFETY PRECAUTIONS

- **This kit is for in vitro use only.** Follow the working instructions carefully. GA GENERIC ASSAYS GmbH and its authorized distributors shall not be liable for damages indirectly or consequentially brought about by changing or modifying the procedure indicated. The kit should be performed by trained technical staff only.
- The expiration dates stated on the respective labels are to be observed. The same relates to the stability stated for reconstituted reagents.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept at 2 - 8 °C before use in the original shipping container.
- Some of the reagents contain small amounts of Thimerosal (< 0.1 % w/v) and Kathon (1.0 % v/v) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- Source materials derived from human body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed:
  - Do not smoke, eat or drink while handling kit material,
  - Always use protective gloves,
  - Never pipette material by mouth,
  - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.