

Please use only the valid version of the package insert provided with the kit.

NAME AND INTENDED USE

c-ANCA (Anti-PR3) is an indirect solid phase enzyme immunoassay (ELISA) for the quantitative measurement of IgG class autoantibodies against proteinase 3 (PR3) in human serum or plasma. The assay is intended for in vitro diagnostic use only as an aid in the diagnosis of certain autoimmune vasculitides such as Wegener's granulomatosis.

SUMMARY AND EXPLANATION OF THE TEST

Anti-neutrophil cytoplasmic antibodies (ANCA) represent a group of autoantibodies directed towards the cytoplasmic components of the neutrophil granulocytes and monocytes. The classical methods for the determination of ANCA are immunofluorescence methods. With these indirect immunofluorescence techniques two main patterns are recognised, a cytoplasmic (cANCA) and a perinuclear (pANCA) type.

The main antigen for the cANCA is the proteinase 3 (PR3), which is a serine proteinase present in primary granules. Antibodies of pANCA positive sera are mainly directed to myeloperoxidase (MPO). Antibodies to other antigens e.g. Lactoferrin, Elastase, Cathepsin-G and also Lysozyme often result in a similar pANCA pattern. Besides, different untypical variants of pANCA IF patterns – granulocyte specific antinuclear antibodies (GS-ANA) – are indistinguishable from pANCA. This makes a clear interpretation and classification of the IF patterns difficult. Therefore every positive IF-ANCA findings esp. pANCA should be differentiated by ELISA techniques using purified antigens.

A survey of documented clinical indications of specific ANCA is given in the table below. PR3-ANCA and MPO-ANCA are reliable serologic markers in the diagnostics of vasculitides. PR3 is the classical autoantigen in Wegener's granulomatosis with a clinical specificity of more than 95%.

cANCA is documented to be present in different diseases. The target antigen myeloperoxidase is mainly present (70%) in microscopic polyangiitis.

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	IFF 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
Morbus Crohn	p-ANCA, atypical ANCA	and other unknown
Chronical Hepatitis		antigens

PRINCIPLE OF THE TEST

Highly purified Proteinase 3 (PR3) is bound to microwells. Antibodies against this antigen, if present in diluted serum or plasma, bind to the respective antigen. Washing of the microwells removes unspecific serum and plasma components. Horseradish peroxidase (HRP) conjugated anti-human IgG immunologically detect the bound patient antibodies forming a conjugate/antibody/antigen complex. Washing of the microwells removes unbound conjugate. An enzyme substrate in the presence of bound conjugate hydrolyses to form a blue colour. The addition of an acid stops the reaction forming a yellow end-product. The intensity of this yellow colour is measured photometrically at 450 nm. The amount of colour is directly proportional to the concentration of IgG antibodies present in the original sample.



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WARNINGS AND PRECAUTIONS

1. All reagents of this kit are strictly intended for in vitro diagnostic use only.
2. Do not interchange kit components from different lots.
3. 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.
4. Avoid contact with the TMB (3,3',5,5'-Tetramethyl-benzidine). If TMB comes into contact with skin, wash thoroughly with water and soap.
5. Avoid contact with the stop solution which is acid. If it comes into contact with skin, wash thoroughly with water and seek medical attention.
6. Some kit components (i.e. controls, sample buffer and buffered wash solution) contain Sodium Azide as preservative. Sodium Azide (NaN_3) is highly toxic and reactive in pure form. At the product concentrations (0.09%), though not hazardous. Despite the classification as non-hazardous, we strongly recommend using prudent laboratory practices (see 8., 9., 10.).
7. Some kit components contain Proclin 300 as preservative. When disposing reagents containing Proclin 300, flush drains with copious amounts of water to dilute the components below active levels.
8. Wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.
9. Do not pipette by mouth.
10. Do not eat, drink, smoke or apply makeup in areas where specimens or kit reagents are handled.
11. Avoid contact between the buffered Peroxide Solution and easily oxidized materials; extreme temperature may initiate spontaneous combustion.

Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. During handling of all kit reagents, controls and serum samples observe the existing legal regulations.

CONTENTS OF THE KIT

Package size	96 determinations
Qty.1	Divisible microplate consisting of 12 modules of 8 wells each, coated with highly purified PR3. Ready to use.
6 vials, 1.5 ml each	Combined calibrators with IgG class Anti- PR3 antibodies (A-F) in a serum/buffer matrix (PBS, BSA, NaN_3 <0.1% (w/w)) containing: IgG: 0; 5; 10; 20; 40; and 100 U/ml. Ready to use.
2 vials, 1.5 ml each	Anti-PR3 controls in a serum/buffer matrix (PBS, BSA, NaN_3 <0.1% (w/w)) positive (1) and negative (2), for the respective concentrations see the enclosed QC insert. Ready to use.

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1 vial, 20 ml	Sample buffer (Tris, NaN ₃ <0.1% (w/w)), yellow, concentrate (5x).
1 vial, 15 ml	Enzyme conjugate solution (PBS, Proclin 300 <0.5% (v/v)), (light red) containing polyclonal anti-human IgG; labelled with horseradish peroxidase. Ready to use.
1 vial, 15 ml	TMB substrate solution. Ready to use.
1 vial, 15 ml	Stop solution (contains acid). Ready to use.
1 vial, 20 ml	Wash solution (PBS, NaN ₃ <0.1% (w/w)), concentrate (50x).

STORAGE AND STABILITY

1. Store the kit at 2 °C - 8 °C.
2. Keep microplate wells sealed in a dry bag with desiccants.
3. The reagents are stable until expiration of the kit.
4. Do not expose test reagents to heat, sun or strong light during storage and usage.
5. Diluted sample buffer and wash buffer are stable for at least 30 days when stored at 2 °C - 8 °C.

MATERIALS REQUIRED**Equipment**

- Microplate reader capable of endpoint measurements at 450 nm
- Multi-Channel Dispenser or repeatable pipette for 100 µl
- Vortex mixer
- Pipettes for 10 µl, 100 µl and 1000 µl
- Laboratory timing device
- Data reduction software

Preparation of reagents

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

SPECIMEN COLLECTION, STORAGE AND HANDLING

- Collect whole blood specimens using acceptable medical techniques to avoid hemolysis.
- Allow blood to clot and separate the serum by centrifugation.
- Test serum should be clear and non-haemolysed. Contamination by hemolysis or lipemia is best 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.

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- Avoid repetitive freezing and thawing of serum samples. This may result in variable loss of autoantibody activity.
- Testing of heat-inactivated sera is not recommended.

PROCEDURAL NOTES

1. Do not use kit components beyond their expiration dates.
2. Do not interchange kit components from different lots.
3. All materials must be at room temperature (20-28 °C).
4. Have all reagents and samples ready before start of the assay. Once started, the test must be performed without interruption to get the most reliable and consistent results.
5. Perform the assay steps only in the order indicated.
6. Always use fresh sample dilutions.
7. Pipette all reagents and samples into the bottom of the wells.
8. To avoid carryover contamination, change the tip between samples and different kit controls.
9. It is important to wash microwells thoroughly and remove the last droplets of wash buffer to achieve best results.
10. All incubation steps must be accurately timed.
11. Control sera or pools should routinely be assayed as unknowns to check performance of the reagents and the assay.
12. Do not re-use microplate wells.

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.



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PREPARATION OF REAGENTS

Preparation of sample buffer

Dilute the contents of each vial of the sample buffer concentrate (5x) with distilled or deionised 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 wash solution

Dilute the contents of each vial of the buffered wash solution concentrate (50x) with distilled or deionised 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.

Sample preparation

Dilute all samples **1:100** with sample buffer before assay.

Therefore combine 10 µl of sample with 990 µl of sample buffer in a polystyrene tube. Mix well.

Controls are ready to use and need not be diluted.

TEST PROCEDURE

1. Prepare a sufficient number of microplate modules to accommodate controls and prediluted patient samples.
2. Pipet **100 µl** of calibrators, controls and prediluted patient samples in duplicate into the wells.

	1	2	3	4	5	6
A	SA	SE	P 1	P 5		
B	SA	SE	P 1	P 5		
C	SB	SF	P 2	P..		
D	SB	SF	P 2	P..		
E	SC	C1	P 3			
F	SC	C1	P 3			
G	SD	C2	P 4			
H	SD	C2	P 4			

SA-SF: standards A to F
P1, P2....: patient sample 1, 2
...
C1: positive control
C2: negative control

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3. Incubate for 30 minutes at room temperature (20-28 °C).
4. Discard the contents of the microwells and wash **3 times** with **300 µl** of wash solution.
5. Dispense **100 µl** of enzyme conjugate into each well.
6. Incubate for 15 minutes at room temperature.
7. Discard the contents of the microwells and wash **3 times** with **300 µl** of wash solution.
8. Dispense **100 µl** of TMB substrate solution into each well.
9. Incubate for 15 minutes at room temperature.
10. Add **100 µl** of stop solution to each well of the modules and incubate for 5 minutes at room temperature.
11. Read the optical density at 450 nm and calculate the results. Bi-chromatic measurement with a reference at 600-690 nm is recommended.

The developed colour is stable for at least 30 minutes. Read optical densities during this time.

Automation

The c-ANCA ELISA is suitable for use on open automated ELISA processors. The test procedure detailed above is appropriate for use with or without automation.

INTERPRETATION OF RESULTS***Quality Control***

This test is only valid if the optical density at 450 nm for Positive Control (1) and Negative Control (2) as well as for the Calibrators A and F complies with the respective range indicated on the Quality Control Certificate enclosed to each test kit! If any of these criteria is not met, the results are invalid and the test should be repeated.

Calculation of results

For Anti-PR3 a 4-Parameter-Fit with lin-log coordinates for optical density and concentration is the data reduction method of choice.

Recommended Lin-Log Plot

First calculate the averaged optical densities for each calibrator well. Use lin-log graph paper and plot the averaged optical density of each calibrator versus the concentration. Draw the best fitting curve approximating the path of all calibrator points. The calibrator points may also be connected with straight line segments. The concentration of unknowns may then be estimated from the calibration curve by interpolation.

Calculation example

The figures below show typical results for c-ANCA (PR3) ELISA. These data are intended for illustration only and should not be used to calculate results from another run.

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Calibrators									
No	Position	OD 1	OD 2	Mean	Conc. 1	Conc. 2	Mean	decl. Conc.	CV %
ST1	A 1/A 2	0,040	0,043	0,042	0,1	0,1	0,1	0.0	5
ST2	B 1/B 2	0,303	0,304	0,303	6,7	6,7	6,7	6,3	0
ST3	C 1/C 2	0,445	0,438	0,442	11,8	11,6	11,7	12,5	1
ST4	D 1/D 2	0,747	0,733	0,74	26	25	26	25	1
ST5	E 1/E 2	1,110	1,135	1,123	49	51	50	50	2
ST6	F 1/F 2	1,623	1,626	1,624	99	100	100	100	0

Interpretation of results

In a normal range study with serum samples from healthy blood donors the following ranges have been established with the Anti- PR3 tests:

<u>Anti-PR3 Ab [U/ml]</u>	
normal:	< 5
elevated:	> 5

Positive results should be verified concerning the entire clinical status of the patient. Also every decision for therapy should be taken individually. It is recommended that each laboratory establishes its own normal and pathological ranges of serum Anti-PR3. The values above should be regarded as guidelines only.

PERFORMANCE CHARACTERISTICS

Parallelism

In dilution experiments sera with high IgG-antibody concentrations were diluted with sample buffer and assayed in the Anti-PR3 kit.

Sample	Dilution	Observed [U/ml]	Expected [U/ml]	Observed/Expected [%]
1	1:100	78.9		
	1:200	39.8	39.5	101
	1:400	20.6	19.7	105
	1:800	10.6	9.9	107
	1:1600	5.3	4.9	108
2	1:100	77.5		
	1:200	37.4	38.8	96
	1:400	19.1	19.4	98
	1:800	9.7	9.7	100
	1:1600	5.0	4.8	104

Precision (Reproducibility)

Statistics for coefficients of variation (CV) were calculated for each of three samples from the results of 24 determinations in a single run for Intra-Assay precision. Run-to-run precision was calculated from the results of 5 different runs with 6 determinations of each sample:

Intra-Assay		
Sample No	Mean (U/ml)	CV (%)
1	10.9	4.7
2	24.6	2.8
3	58.5	2.8

Inter-Assay		
Sample No	Mean (U/ml)	CV (%)
1	10.4	6.2
2	23.4	8.8
3	60.7	3.9

Sensitivity

The lower detection limit for Anti-PR3 has been determined at 0.5 U/ml.

Specificity

The microplate for Anti-PR3 is coated with highly purified proteinase 3 (PR3), isolated from human granulocytes showing high specific proteinase activity. The test kit is specific only for autoantibodies directed to the respective antigen. No cross reactivities have been observed.

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Since no international reference preparation for Anti-PR3 autoantibodies is available, the assay system is calibrated in relative arbitrary units.

LIMITATIONS OF PROCEDURE

The Anti-PR3 ELISA is a diagnostic aid and by itself is not diagnostic. 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.

INTERFERING SUBSTANCES

No interference has been observed with haemolytic (up to 1000 mg/dL), lipemic (up to 3 g/dL triglycerides) or bilirubin (up to 40 mg/dL) containing sera.

Nor have any interfering effects been observed with the use of anticoagulants.

However for practical reasons it is recommended that grossly haemolysed or lipemic samples should be avoided.

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