



RUO in the USA

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PRINCIPLE OF THE PROCEDURE

ANCA Screen is an indirect solid phase enzyme immunometric assay (ELISA). It is designed for the qualitative screening of IgG class autoantibodies directed against PR3 and MPO antigens. The antigens were coated in microplates, which can be divided into 96 wells or can be used complete for 96 determinations. During this procedure the binding of present autoantibodies, as well as the formation of the sandwich complexes and enzymatic colour 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 immobilised 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 recognise the autoantibodies bound to the immobilised 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 colour of the solutions change into blue. Adding 1 M hydrochloric acid as stop solution stops colour development. The solutions colour 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

Anti-neutrophil cytoplasmic antibodies (ANCA) represent a group of autoantibodies directed towards the cytoplasmic components of the neutrophilic granulocytes and monocytes. The classical methods for the determination of the ANCAs are the immunofluorescent methods. With indirect immunofluorescence techniques two main patterns are recognized.

- a cytoplasmic (c-ANCA) pattern
- and a perinuclear (p-ANCA) type

Recently, the main antigens for the c- and p-ANCAs have been identified. The target antigen for 80-90 % of c-ANCA antibodies is the proteinase 3 (PR3), a serine proteinase from a-granules. 10-20 % of c-ANCAs are directed to other proteins. The solubilization of ethanol-fixed granulocytes causes a binding of positively charged proteins around the nucleus. Antibodies to these proteins appear in the immunofluorescence as p-ANCAs. Approx. 90 % of p-ANCA positive sera contain autoantibodies directed to myeloperoxidase (MPO) which is located in the granula of neutrophilic granulocytes. Antibodies to other antigens e.g. lactoferrin, elastase, cathepsin G and lysozyme often result in a similar p-ANCA patterns. Beside different untypical variants of p-ANCA, IF patterns of granulocyte specific antinuclear antibodies (GS-ANA) are indistinguishable from those of p-ANCAs. A distinct interpretation and classification of the IF patterns is quite difficult. Therefore every positive IF-ANCA finding esp. p-ANCAs should be differentiated by ELISA techniques



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using purified antigens. The anti-PR3 antibody titer correlates well with the clinical status of the disease. Antibody titers are decreasing under therapy and become negative after remission. Anti-MPO levels correlate with the clinical status too. They are always higher during the active disease than after remission. A survey of documented clinical indications of specific ANCAs is given in the following table. Anti-PR3 and anti-MPO antibodies are reliable serological markers for the diagnosis of vasculitides. PR3 is the classical autoantigen in Wegener’s granulomatosis with a clinical specificity of more than 95%. p-ANCAs are documented to be present in 70% of patients with Microscopic Polyangiitis.

	IFA patterns	Target antigen
Systemic Vasculitic Syndromes		
Wegener’s Granulomatosis	c-ANCA, rarely p-ANCA	PR3, rarely MPO
Microscopic Polyangiitis	c-ANCA, p-ANCA	PR3, MPO
Churg-Strauss-Syndrome	p-ANCA	MPO
Rapidly progressive Glomerulonephritis	p-ANCA	MPO
Polyarthritis nodosa	Rarely ANCA	Rarely PR3 and MPO
Unclassified Vasculitis	Rarely	No PR3 and MPO
Collagen Diseases and other Rheumatic Disorders		
Rheumatoid arthritis	GS-ANA, p-ANCA, atypical ANCA	Unknown, ANA, Rarely MPO, Lactoferrin
SLE	p-ANCA	Rarely MPO, Lactoferrin
Other Diseases		
Ulcerative Colitis		Cathepsin G, Lactoferrin
Morbus Crohn	p-ANCA, atypical ANCA	and other unknown
Chronic Hepatitis		Antigens

SPECIFICITY

The microplate is coated with a mixture of PR3 and MPO antigens, highly purified by affinity chromatography. The ANCA screen test is specific only for autoantibodies directed to these antigens. No cross-reactivities 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:



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

MATERIALS SUPPLIED

Package size	96 determ.
Divisible microplate consisting of 12 modules of 8 wells,	1
coated with a mixture of highly purified antigens PR3 and MPO:	
ANCA controls in a PBS/BSA matrix:	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

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MATERIALS REQUIRED

Equipment

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

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 μ 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 μ l of sample may be diluted with 1000 μ 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.



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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 contaminations change 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.

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.

	1	2	3	4	5	6	
A	C1	P3					
B	C1	P4					C1 .. C3 = Control 1 .. 3
C	C2	P5					P1..P.. = Patient Sample
D	C2	P6					
E	C3	P..					
F	C3						
G	P1						
H	P2						

2. Pipette 100 µl of controls and prediluted patient samples into the wells.
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 solution 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 leave untouched for 5 minutes.
11. Read the optical density at 450 nm and calculate the results. Bi-chromatic measurement with reference at 600-650 nm is recommended.

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



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CALCULATION OF RESULTS

Evaluation of the ANCA screen test is easily carried out by direct comparison of the optical density of each patient sample with the optical density of the cut-off control.

Patient samples exhibiting optical densities higher than the optical density of the cut-off control are considered to be positive.

Negative: OD Patient < OD cut-off control
 Positive: OD Patient ≥ OD cut-off control

For detailed quantification of the results, the “Index Value” can express each patient-OD value. The Index Value is calculated by dividing the patient-OD by the cut-off-OD.

**ANCA Screen
 (Index Value)**
Negative: < 1.0
Positive: ≥ 1.0

The calculation of Index Values is not influenced by variations of the sample-OD and/or Cut-Off-OD. Index Values are recommended for long-term validations (i.e. internal quality control samples).

Using the quantitative Anti-PR3 and Anti-MPO ELISA should carry out further differentiation and typing.

CALCULATION EXAMPLE

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

Sample No.	Extinction	Cut-Off Extinction	Index Value	Interpretation
1	0.107	0.535	0.2	Negative
2	0.735	0.535	1.4	Positive
3	1.294	0.535	2.4	Positive
4	2.496	0.535	4.7	Positive

ASSAY CHARACTERISTICS

PRECISION

Statistics for Coefficients of variation (CV) were calculated for each of three samples, specific for MPO or PR3, from the results of 24 determinations in a single run for Intra-Assay precision. Run-to-run precision was calculated from the results of 3 different sera, with different MPO or PR3 antibody titres, on 6 microplates with 8 determinations of each sample:

Intra-Assay		
Sample No.	Mean (U/ml)	CV [%]
A1 (MPO)	12	7.1
A2 (PR-3)	20	3.5
B1 (MPO)	47	6.3
B2 (PR-3)	52	4.2
C1 (MPO)	87	6.7
C2 (PR-3)	95	5.8

Inter-Assay		
Sample No.	Mean (Index Value)	CV [%]
A1 (MPO)	15	4.3
A2 (PR-3)	22	4.7
B1 (MPO)	50	7.2
B2 (PR-3)	50	6.8
C1 (MPO)	85	5.5
C2 (PR-3)	91	6.9

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