DRG® ANCA Screen (EIA-3776)

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Revised 30 Dec. 2008 (Vers. 2.1) IVD

NAME AND INTENDED USE

The ANCA Screen assay is a qualitative enzyme immunoassay (EIA) intended to screen for the presence of IgG class autoantibodies against PR3 and MPO in human serum or plasma as an aid in the determination of systemic vasculitides.

SUMMARY AND EXPLANATION OF THE TEST

Anti-neutrophil cytoplasmic antibodies (ANCA) represent a group of autoantibodies directed towards the cytoplasmatic 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 cytoplasmatic (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 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.

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	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,	unknown, ANA,
	atypical ANCA	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

PRINCIPLE OF THE TEST

A mixture of highly purified PR3 and MPO antigens is bound to microwells. Antibodies against these antigens, if present in diluted serum or plasma, bind to the respective antigens. Washing of the microwells removes unspecific serum and plasma components. Horseradish peroxidase (HRP) conjugated anti-human IgG immunologically detects 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 hydrolyzes to form a blue color. The addition of an acid stops the reaction forming a yellow end-product. The intensity of this yellow color is measured photometrically at 450nm.
The amount of colour is directly proportional to the concentration of IgG antibodies present in the original sample.

WARNINGS AND PRECAUTIONS

1. All reagents of this kit are strictly intended for in vitro use only. In the United States, this kit is intended for Research 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, 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 a mixture of highly purified antigens, PR3 and MPO. Ready to use.
3 vials, 1.5 ml each	Anti-ANCA **controls** in a serum/buffer matrix (PBS, NaN_3 <0.1% (w/w)). Negative Control (A), Cut-Off Control (B) and Positive Control (C). Ready to use.
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 rabbit 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).

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STORAGE AND STABILITY

1. Store the kit at 2-8 °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 2-8 °C.

MATERIALS REQUIRED

Equipment

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

Preparation of reagents

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

SPECIMEN COLLECTION, STORAGE AND HANDLING

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

PREPARATION OF REAGENTS

Preparation of sample buffer
Dilute the contents of each vial of the sample buffer concentrate (5x) with distilled or deionized 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 deionized 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 patient 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.

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TEST PROCEDURE

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

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

C1 Negative Control
C2 Cut-Off Control
C3 Positive Control
P... Patient Sample

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.

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INTERPRETATION OF RESULTS

Quality Control

This test is only valid if the optical density at 450 nm for Negative Control (A), Cut-Off Control (B) and Positive Control (C) complies with the respective range indicated on the Quality Control Certificate enclosed to each test kit !
If any of these criteria is not fulfilled, the results are invalid and the test should be repeated.

Qualitative interpretation 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

Semi-quantitative interpretation of results

For detailed quantification of the results, each patient-OD value can be expressed by the "Index Value".
The Index Value is calculated by dividing the patient-OD by the Cut-Off-OD.

$$\text{Index value} = \frac{OD_{\text{Sample}}}{OD_{\text{Cut-off}}}$$

	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). The calculation of Index Values is not influenced by variations of the sample-OD and/or Cut-Off-OD.
Further differentiation and typing should be carried out by using the quantitative Anti-PR3 and Anti-MPO ELISA.

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PERFORMANCE 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 (Index Value)	CV [%]
A1 (MPO)	0.9	9.1
A2 (PR-3)	1.8	5.5
B1 (MPO)	2.5	6.3
B2 (PR-3)	3.1	7.2
C1 (MPO)	4.7	6.7
C2 (PR-3)	4.9	5.8

	Inter-Assay	
Sample No.	Mean (Index Value)	CV [%]
A1 (MPO)	1.1	6.3
A2 (PR-3)	1.6	8.7
B1 (MPO)	2.3	7.2
B2 (PR-3)	2.8	9.8
C1 (MPO)	3.6	5.5
C2 (PR-3)	4.1	6.9

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 anti-PR3 and anti-MPO autoantibodies are available, the assay is calibrated in relative arbitrary units.

LIMITATIONS OF PROCEDURE

The ANCA Screen ELISA 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.

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 hemolyzed or lipemic samples should be avoided.

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