ORGENTEC Diagnostika GmbH
Carl-Zeiss-Straße 49
55129 Mainz - Germany

Phone: +496131/92580
Fax: +496131/925858
Internet : www.orgentec.com

Instruction for use
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ORG 589 ANCAscreen

Immunometric Enzyme Immunoassay for the qualitative screening of IgG autoantibodies to PR3 and MPO

For research use only. Not for use in diagnostic procedures.

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NAME AND INTENDED USE

The ORGENTEC ANCAscreen 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. For research use only, not for use in diagnostic procedures.

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 colour. The addition of an acid stops the reaction forming a yellow end-product. The intensity of this yellow colour 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 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 (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 by assaying con-trols 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 determ.
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).

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 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

COLLECTION, STORAGE AND HANDLING OF UNKNOWNS

1. Collect whole blood 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. Unknowns 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 contamination, change the tip between samples and different kit con-
   trols.
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 1:101 with sample buffer before assay. Therefore combine 10 µl of sample with 1000 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 predi-luted samples.
2. Pipette **100 µl** of controls and prediluted samples in duplicate into the wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**C1**	**P3**										
B	**C1**	**P4**										
C	**C2**	**P5**										
D	**C2**	**P6**										
E	**C3**	**P...**										
F	**C3**											
G	**P1**											
H	**P2**											

C1 Negative Control
C2 Cut-off Control
C3 Positive Control
P... Samples

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.

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.

Evaluation of the ANCAscreen test is easily carried out by direct comparison of the optical density of each unknown with the optical density of the Cut-Off Control. Unknowns exhibiting optical densities higher than the optical density of the Cut-Off Control are considered to be positive.

Negative: OD Sample	**<**	OD Cut-Off Control
Positive: OD Sample	≥	OD Cut-Off Control

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

$$\text{Index Value} = \frac{OD_{Sample}}{OD_{Cut-Off}}$$

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. Index Values are recommended for long term validations (i.e. internal quality control samples).

Further differentiation and typing should be carried out by using the quantitative Anti-PR3 and Anti-MPO ELISA.

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				Inter-Assay		
Sample No	Mean (Index Value)	CV [%]		Sample No	Mean (Index Value)	CV [%]
A1 (MPO)	0.9	9.1		A1 (MPO)	1.1	6.3
A2 (PR3)	1.8	5.5		A2 (PR3)	1.6	8.7
B1 (MPO)	2.5	6.3		B1 (MPO)	2.3	7.2
B2 (PR3)	3.1	7.2		B2 (PR3)	2.8	9.8
C1 (MPO)	4.7	6.7.		C1 (MPO)	3.6	5.5
C2 (PR3)	4.9	5.8		C2 (PR3)	4.1	6.9

Specificity

The microplate is coated with a mixture of PR3 and MPO antigens, highly purified by affinity chromatography. The ANCAscreen 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 ANCAscreen ELISA is a research tool.

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 hemolysed or lipemic samples should be avoided.

INCUBATION SCHEME

1. Pipet **100 µl** calibrator, control or patient sample
   → Incubate for **30 minutes** at room temperature
   → Discard the contents of the wells and wash 3 times with **300 µl** wash solution
2. Pipet **100 µl** enzyme conjugate
   → Incubate for **15 minutes** at room temperature
   → Discard the contents of the wells and wash 3 times with **300 µl** wash solution
3. Pipet **100 µl** substrate solution
   → Incubate for **15 minutes** at room temperature
4. Add **100 µl** stop solution
   → Leave untouched for **5 minutes**
   → Read at **450 nm**