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Instruction for use
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ORG 524 Anti-Elastase

Immunometric Enzyme Immunoassay for the quantitative determination of autoantibodies against Elastase

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NAME AND INTENDED USE

Anti-Elastase is an indirect solid phase enzyme immunoassay (ELISA) for the quantitative measurement of IgG class autoantibodies against Elastase in human serum or plasma. The assay is intended for in vitro diagnostic use only.

SUMMARY AND EXPLANATION OF THE TEST

The acronym ANCA (Antineutrophil Cytoplasmic Autoantibodies) is defined by an accumulation of autoantibodies with specificity against different granulocytic, monocyctic and probably endothelial cytoplasmic antigens.

PR3 and MPO are well defined as reliable serological markers for a definite group of primary systemic vasculitides (PSV), which were also named ANCA associated vasculitides (AAV). The occurrence of AAV is clearly higher than supposed. The incidence is 1.5 per 1000 and in the group of older persons nearly 5 per 1000. The clinical appearance of the AAV is characterised through manifestations in lung, kidney and respiratory tract. In the last years, newer investigations discovered and characterised a couple of new pANCA antigens: Elastase, Cathepsin G, Lysozyme and Lactoferrin.

Up to now, ANCA screening has been done with immunofluorescence techniques, but often there have been difficulties in the evaluation and in clinical findings. Therefore, the results have to be scrutinised with counter examinations on other cells or in other test systems like ELISA. Moreover it was not possible to differentiate the single cANCA and pANCA antigens.

Proteinase 3

The major antigen for the cANCA reactivity is the neutral serin protease 3 (synonyms: p29, AGP7, Wegener autoantigen), which belongs to the Trypsin/Chymotrypsin family. In 1988 several groups showed that the antigen is a protein with a molecular weight of 29 kDa. PR3 was already described in 1973 by Ohlsson and Olsson under the name neutrophile collagenase. In the meantime it seems certain, that autoantibodies against PR3 are highly specific as serological marker for the diagnosis of Wegener's granulomatosis (specificity: initial phase 50%, generalisation phase > 90%). Moreover there is a correlation between the concentration of the autoantibodies and the disease activity.

Myeloperoxidase

Myeloperoxidase is the major antigen in nearly 60% of the pANCA findings. The occurrence of autoantibodies against MPO is classified as relevant marker for the rapid progressive nephritis. Moreover these antibodies occur in 70-90% in all patients with serious kidney injury. Over and about they have also been detected at the Churg-Strauß-Syndrom (CSS), Microscopic Polyangiitis (MPA) and other vasculitis diseases. The concentration of the autoantibodies correlates well with the disease activity of MPA. MPA is also characterised by clinical manifestations of lung, kidney and respiratory tract, but these manifestations are, in contrast to WG, not granulomatous. However, these antibodies have, in contrast to the high specificity of PR3 antibodies for WG, a minor specificity of 60% in the diagnosis of MPA. The absence of autoantibodies against MPO and PR3, by simultaneous detection of ANA can be used as a tool for differential diagnosis between AAV and SLE induced vasculitis.

BPI

Bactericidal permeability-increasing protein, BPI is a membrane-located protein that is classed as an ANCA-Antigen of polymorph-nuclear granulocytes and monocytes that bind endotoxin.

Its autoantibodies are now classified as cANCA. Due to BPIs high affinity to lipopolysaccharides its anti-microbial effect against Gram-negative bacteria is significant. BPI is splitted and thus inactivated by using elastase or other serine protease. Autoantibodies against BPI are above all detected in chronically infectious intestinal diseases such as Morbus Crohn or colitis ulcerosa. In contrast to anti-MPO and anti-PR3 autoantibodies, those against BPI seem not to have any association with vasculitis.

Elastase

Elastase is a serine protease with a sequence homology of 54% to that of proteinase 3. It occurs mainly in polymorph-nuclear neutrophilic granulocytes (PMN), in macrophages and endothelial cells. The dismantling of proteoglycans by neutrophiles is mainly due to elastase' proteolytic activity. Furthermore, elastase participates decisively in tissue destruction connected with emphysemas and rheumatoid arthritis. Autoantibodies against this antigen are generally associated with inflammatory rheumatic disorders, e.g. rheumatoid arthritis and vasculitis.

Cathepsin G

The cathepsins belong to a group of intracellular proteases mainly found in lysosomes, especially of the spleen, the liver and the kidney. Cathepsin G is a serine protease and a further pANCA antigen. It participates to a great part in the destruction of osteid tissue as of its hydrolytic properties. The autoantibodies against Cathepsin G occur mainly in collagenosis and other related inflammatory rheumatic diseases, e.g. SLE, Sjögren syndrome and Felty syndrome.

Lysozyme

Lysozyme is a glykosidase, which decomposes the glycosidic bond between C-1 of MNAc and C-4 of GlcNAc. Lysozyme is localised in the azurophilic as well as in the specific granules of neutrophiles and in extracellular liquid compartments like tears and salivary, where it spreads out his antimicrobial activities against invading bacteria. LZ belongs also to the pANCA and autoantibodies against Lysozyme occur in higher frequency in rheumatoid vasculitis and inflammatory bowel disease like colitis ulcerosa.

Lactoferrin

Lactoferrin (LF) is an iron-binding protein, which occurs in high concentrations in secretions at mucosa surfaces, in tears and in milk. LF also resides in the specific granules of polymorph-nuclear neutrophil leukocytes (PMN) and becomes exocytosed upon PMN activation. During active inflammatory disease, raised serum levels of LF can be measured. The physiological anti microbial effect of Lactoferrin depends on its iron-binding capacity, because most of the bacteria require iron for their own physiological pathways. LF inhibits myelopoiesis, prevents complement activation and prevents the formation of hydroxyl radicals. It is quite possible that LF has several important roles, like secretory IgA, as a non-specific antiphlogistic defence factor at mucosal surfaces. LF belongs to the pANCA, depending on the redistribuiton from the granules toward the nuclei, upon ethanol fixation. Autoantibodies against Lactoferrin occur in higher frequency in patients with rheumatoid vasculitis (RV), colitis ulcerosa (CU) and primary sclerosing cholangitis (PSC).

PRINCIPLE OF THE TEST

Highly purified Elastase is bound to microwells. Antibodies against this antigen, 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 450 nm. 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 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 and 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 hydrochloric acid (1 M). 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 determ.
Qty.1	Divisible microplate consisting of 12 modules of 8 wells each, coated with highly purified Elastase. Ready to use.
6 vials, 1.5 ml each	combined Calibrators with IgG class Anti-Elastase (A-F) in a serum/buffer matrix (PBS, BSA, NaN ₃ <0,1% (w/w)) containing: IgG: 0; 6.3; 12.5; 25; 50; and 100 U/ml. Ready to use.
2 vials, 1,5 ml each	Anti-Elastase 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 package insert. 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 (1 M hydrochloric 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 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 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.

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.

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	P1	P5		
B	SA	SE	P1	P5		
C	SB	SF	P2	P..		
D	SB	SF	P2	P..		
E	SC	C1	P3			
F	SC	C1	P3			
G	SD	C2	P4			
H	SD	C2	P4			

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

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 ORGENTEC Anti-Elastase 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 Calibrator 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 fulfilled, the results are invalid and the test should be repeated.

Calculation of results

For Anti-Elastase IgG 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 Anti-Elastase ELISA. These data are intended for illustration only and should not be used to calculate results from another run.

Calibrators									
No	Position	OD 1	OD 2	Mean	Conc. 1	Conc. 2	Mean	decl. Conc.	CV%
STA	A 1 / B 1	0.036	0.037	0.037	0.1	0.1	0.1	0.0	3
STB	C 1 / D 1	0.423	0.425	0.424	6.2	6.2	6.2	6.3	0
STC	E 1 / F 1	0.716	0.744	0.730	12.5	13.2	12.9	12.5	3
STD	G 1 / H 1	1.101	1.132	1.116	24	25	24	25	2
STE	A 2 / B 2	1.660	1.637	1.649	51	50	51	50	1
STF	C 2 / D 2	2.137	2.151	2.144	99	101	100	100	0

Interpretation of results

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

	Anti-Elastase IgG [U/ml]
normal:	< 10
elevated:	≥ 10

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-Elastase antibodies. The above reference ranges should be regarded as guidelines only.

PERFORMANCE CHARACTERISTICS

Parallelism

In dilution experiments sera with high antibody concentrations were diluted with sample buffer and assayed in the Anti-Elastase kit. The assay showed linearity over the full measuring range.

Sensitivity

The lower detection limit for the Anti-Elastase test was determined at 0.5 U/ml.

Specificity

The microplate is coated with Elastase. The antigen preparation is highly purified by affinity chromatography. The Anti-Elastase test is specific only for autoantibodies directed against anti-Elastase. No crossreactivities to the other ANCA antigens have been observed.

Calibration

Since no international reference preparations for Anti-Elastase autoantibodies is available, the assay system is calibrated in arbitrary units.

LIMITATIONS OF PROCEDURE

The Anti-Elastase 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.

REFERENCES

1. Hauschild, S.; Schmitt, W.H.; Csernok, E.; Flesch, B. K.; Rautmann, A.; Gross, W.L.. ANCA in systemic vasculitides, collagen vascular diseases, rheumatic disorders and inflammatory bowel diseases. ANCA-associated Vasculitides: Immunological and clinical aspects. Edited by W. L. Gross, Plenum Press, New York, 1993, pp. 245 - 251.
2. Schmitt, W.H.; Csernok, E.; Flesch, B. K.; Hauschild, Gross, W.L.. Autoantibodies directed against Lysozyme: A new target Antigen for anti-neutrophil cytoplasmic antibodies (ANCA). ANCA-associated Vasculitides: Immunological and clinical aspects. Edited by W. L. Gross, Plenum Press, New York, 1993, 267 - 272.
3. Skogh, T. and Peen, E.. Lactoferrin, Anti-Lactoferrin antibodies and inflammatory disease. ANCA-associated Vasculitides: Immunological and clinical aspects. Edited by W. L. Gross, Plenum Press, New York, 1993, pp. 533 - 538.

4. Stoffel, M. P.; Csernok, E.; Herzberg, C.; Johnston, T.; Carroll, S.F. and Gross, W. L.. Anti-neutrophil cytoplasmic antibodies (ANCA) directed against bactericidal/permeability increasing protein (BPI): a new seromarker for inflammatory bowel disease and associated disorders. Clin Exp. Immunol. 1996, No. 104, pp. 54 - 59.
5. Zhao, M. H.; Jayne, D. R. W.; Ardiles, L. G.; Culley, F.; Hodson, M.E. and Lockwood. Autoantibodies against bactericidal /permeability increasing protein in patients with cystic fibrosis. J. Med. 1996, No. 89, pp. 259 - 265.
6. Wiik, A.; Stumman, L.; Kjeldsen, L.; Borregaard, N.; Ullman, S.; Jacobsen, S. and Halberg, P.. The diversity of perinuclear antineutrophil cytoplasmic antibodies (pANCA) antigens. Proceedings of the 6th international ANCA workshop, France - July 1995, pp. 15 - 17.
7. Jenette, J. C. and Falk, R. J.. Antineutrophil cytoplasmic autoantibodies and associated diseases: A review. Am. J. of Kidney Disease 1990, Vol XV, No. 6, pp. 517 - 529.
8. Schmitt, W. H. and Gross, W. L.. Antineutrophile zytoplasmatische Antikörper (ANCA). Internist 1995, No. 36, pp. 282 - 290.
9. Gross, W. L. and Rheinhold-Keller, E.. ANCA-assoziierte Vaskulitiden (Wegener Granulomatose, Churg-Strauß-Syndrom, Mikroskopische Polyangiitis) - 1. Systematik, Pathogenese und Klinik. Z. Rheumatol (1995), No. 54, pp. 279 - 290.
10. de Grot, K.; Schnabel, A. and Gross, W. L.. ANCA-assoziierte Vaskulitiden (Wegener Granulomatose, Churg-Strauß-Syndrom, Mikroskopische Polyangiitis) - 1. Diagnostisches Procedere. Z. Rheumatol (1995), No. 54, pp. 291 - 302.

INCUBATION SCHEME

