

**PRINCIPLE OF THE PROCEDURE**

Anti-Lactoferrin is an indirect solid phase enzyme immunometric assay (ELISA). It is designed for the quantitative determination of anti-neutrophil cytoplasmic antibodies (ANCA) directed against Lactoferrin. The assays recognise IgG class autoantibodies. The microplate is coated with Lactoferrin. The microplate can be divided into 12 modules of 8 wells each or can be used completely for 96 determinations. Each well can be separated from the module ("break-away"). The binding of present autoantibodies, as well as the formation of the sandwich complexes and enzymatic colour reaction takes place during three different reaction phases:

Phase 1:

Calibrators, controls and prediluted patient samples are pipetted into the wells of the microplate. Any present anti-Lactoferrin antibodies bind to the inner surface of the wells. After 30 minutes incubation the microplate is washed with wash solution 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 immobilized antigens. After a 15 minutes incubation any excessive enzyme conjugate, which is not specifically bound is washed away with wash solution.

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. Colour development is stopped by adding 1 M hydrochloric acid as stop solution. The solutions colour change into yellow. The amount of colour is directly proportional to the concentration of IgG present in the original sample. 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. The optical density for each calibrator may be graphically plotted against the concentration of IgG and unknowns extrapolated from the curve.

CLINICAL RELEVANCE

The acronym ANCA (Antineutrophil Cytoplasmic Autoantibodies) is defined by an accumulation of autoantibodies with specificity against different granulocytic, monocyctic and probably endothelial cytoplasmic antigens. Autoantibodies against endocellular components of neutrophil granulocyte (PMN) are known for a longer period. At first, in 1964 they have been described as granulocyte specific factor directed against anti nuclear components (GS-ANA). Under the term ANCA, they were first reported in 1982 in a few patients who had necrotizing glomerulonephritis without immune deposits, but the relevance was still unclear. The next report of ANCA was in 1984 where these autoantibodies have been observed in four patients with systemic vasculitis and three of whom had also necrotizing glomerulonephritis. In 1985, a collaborative study in the Netherlands and Denmark described these factors, showing a high sensitivity for active Wegener's granulomatosis, as APCA (Anti Cytoplasmic Antibodies) and they suggested that APCA could be used as a marker for disease activity. In 1988 further investigations substantiated the association of ANCA with Wegener's Granulomatosis and polyarteritis nodosa, and also noted that ANCA occur in patients with pauci-immune ("idiopathic") necrotizing and crescentic glomerulonephritis who have no evidence for extrarenal disease. Proteinase 3 (PR3), showing a central accentuated pattern (cANCA) has been described as major antigen for Wegener's Granulomatosis. A new pattern, showing a high specificity for the microscopic Polyangiitis, with perinuclear fluorescence (pANCA) was also observed in 1988. At first, only Myeloperoxidase (MPO) was described as antigen. In the last years, newer investigations discovered and characterised a couple of new pANCA antigens: Elastase, Cathepsin G, Lysozyme and Lactoferrin. In the meantime, 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).

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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. 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 serine 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 neutrophil 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 was first isolated by Agner in 1941, who gave it the name "verdoperoxidase", because of its green colour and ability to catalyse peroxidase reactions. It is MPO that gives pus its greenish tone. In nearly 60% of the pANCA findings MPO is the major antigen. 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 over 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 of 55kDa molecular weight and is classed as an ANCA-Antigen of polymorph-nuclear granulocytes and monocytes that bind endotoxin. Its autoantibodies are now classified as cANCA. Due to BPI's 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 neutrophils 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 osseous tissue as of its hydrolytic properties. The autoantibodies against Cathepsin G

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occur mainly in collagenosis and other related inflammatory rheumatic diseases, e.g. SLE, Sjögren syndrome and Felty syndrome.

Lysozyme

In 1922, Lysozyme (LZ) was accidentally discovered by Alexander Flemming. LZ is a glycosidase consisting of 129 amino acid residues with a molecular weight of 14,6 kDa, 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 neutrophils and in extracellular liquid compartments like tears and salivary, where it spreads out its 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, with a molecular weight of 77-93 kDa, which occurs in high concentrations in secretions at mucosa surfaces, in tears and in milk. LF also resides in the specific granules of polymorphonuclear 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 redistribution 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).

NORMAL VALUES

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

	anti-Lactoferrin Ab's [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-Lactoferrin antibodies. The above reference ranges should be regarded as guidelines only.

SPECIFICITY

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

CALIBRATION

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

WARNINGS AND PRECAUTIONS

All reagents of this test kit are strictly intended for in vitro 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

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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:

- 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 oxidized materials; extreme temperatures may initiate spontaneous combustion.

MATERIALS SUPPLIED

Package size	96 determ.
Divisible microplate consisting of 12 modules of 8 wells, 1	
coated with highly purified Lactoferrin	
Anti-Lactoferrin calibrators in a PBS/BSA matrix 6 vials, 1.5 ml each	
containing respectively: 0; 6.3; 12.5; 25; 50 and 100 U/ml	
Anti-Lactoferrin controls in a PBS/BSA matrix..... 2 vials, 1.5 ml each	
(positive and negative), for the respective concentrations	
see the enclosed package insert	
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 two 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)
Calibration range:	6.3 - 100 U/ml
Sensitivity:	0.5 U/ml
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 µl, 100 µl and 1000 µl

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 µl
- data reduction software

SPECIMEN COLLECTION AND PREPARATION

For determination of Anti-Lactoferrin antibodies serum or plasma are the preferred sample matrixes.

All serum and plasma samples are prediluted 1 : 100 with sample buffer. Therefore 10 µl of sample may be diluted with 1000 µl 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 have 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.

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 contamination 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.

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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 calibrators, controls and prediluted patient samples in duplicates.

	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... patient sample 1, 2 ...
C1: positive control
C2: negative control

2. Pipet **100 µl of calibrators, 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 color is stable for at least 30 minutes. Read optical densities during this time.

CALCULATION OF RESULTS

For the Anti-Lactoferrin antibody test a 4-Parameter-Fit with lin-log coordinates for optical density and concentration is recommended. Spline Approximation and log-log coordinates are also suitable.

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.

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CALCULATION EXAMPLE

The figures below show typical results for Anti-Lactoferrin. These data are intended for illustration only and should not be used to calculate results from another run.

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

ASSAY CHARACTERISTICS

Sensitivity

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

Parallelism

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

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