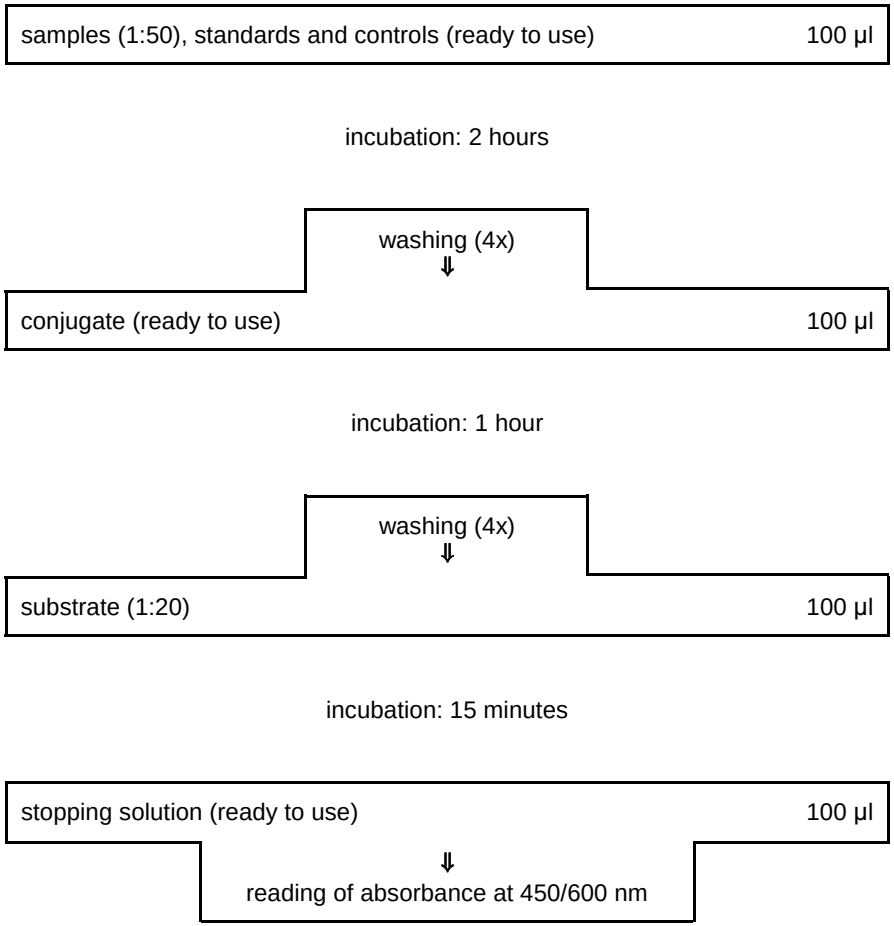
Scheme of the assay



CE

Operating instructions

Anti-MPO (P-ANCA) ELISA

Microtiter plate enzyme immunoassay for the detection and quantification of anti-myeloperoxidase autoantibodies in serum

(for *in vitro* use only)

Cat. No.	:	EA005/96
Determinations	:	96
Storage	:	2˚ - 8˚C

12.10.04

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INTRODUCTION

Since 1985, detection of **a**nti-**n**eutrophil **c**ytoplasm **a**utoantibodies (ANCA) has become a routine diagnostic tool for patients with vasculitis and glomerulonephritis (1-6, 16, 23). Using the indirect immunofluorescence technique (IIF) with ethanol-fixed human neutrophils, different staining pattern are observed. Autoantibodies causing a cytoplasmic pattern (C-ANCA), are generally directed against proteinase 3 (PR3) (8, 9) and can be detected with the Anti-PR3 (C-ANCA) ELISA (Cat.-No.: EA004/96).

In contrast, a perinuclear pattern (P-ANCA) can be produced by a variety of different autoantibody specificities. Originally, it was reported that myeloperoxidase (MPO) is the main target antigen of P-ANCA (3, 4), but later it became evident that only about 10% of P-ANCA findings are due to anti-MPO antibodies (12, 13). A perinuclear staining pattern can also be produced by autoantibodies against elastase, cathepsin G, lactoferrin or lysozyme and even by antinuclear antibodies (e.g. in SLE). Therefore, specific detection of anti-MPO antibodies, one subspecificity of P-ANCA, is only possible by means of an assay like this Anti-MPO (P-ANCA) ELISA, where highly purified myeloperoxidase is used as antigen.

DIAGNOSTIC RELEVANCE

Anti-MPO antibodies are marker for Microscopic polyangiitis as defined on the "Chapel Hill consensus conference" 1994 (17). Microscopic polyangiitis is distinguished from Wegener´s granulomatosis by the obligatory absence of granulomas and the much more frequent kidney involvement. Anti-MPO antibodies are a rare finding in Churg-Strauss syndrome and in Wegener´s granulomatosis (22). Although some studies describe anti-MPO antibodies in patients with Systemic lupus erythematosus, these data were not confirmed by other groups using optimized assays (12, 14, 18, 19).

There are some data indicating that anti-MPO antibodies, like anti-PR-3 antibodies, are marker of disease activity (12,13, 24).

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INTERPRETATION OF RESULTS

Samples with values **lower than 2.5 U/ml** are considered **negative** for Anti-MPO.
Samples with values **between 2.5 U/ml and 5 U/ml** are on the borderline and are considered **equivocal**.
Samples with values **equal to or greater than 5 U/ml** are considered **positive** for Anti-MPO.

If values between 2.5 U/ml and 5 U/ml were determined or if negative results were obtained for patients clinically suspected of vasculitis, periodical follow-up controls are recommended.

LIMITATIONS

Using each of the different measuring systems for autoantibodies (IIF, ELISA, RIA) it is to be considered that in this case a uniform target molecule does not exist. In the patient sera autoantibody mixtures are present (light chains, subclasses, allotypes, epitope specificities) which vary from individual to individual in their composition and therefore also in their avidity independently of the total concentration. The ability to bind to the autoantigen is their only common property that can be used to distinguish them from other antibodies and makes them a entity but it can have individual characteristics. This condition is the reason for patient sera showing the same reaction intensity in a given dilution but giving different results in other dilutions. Despite this limitation, the standard used in this test kit has been proven to be very useful for quantification of anti-MPO antibody activity in a great number of sera. However, if the dilution curve of a serum is found to be considerably different from the standard curve, it might be advantageous to prepare a patient-specific standard from this serum for follow-up determinations of this individual patient.

USEFUL AIDS

The following 0.5 ml vials that can be arranged as microtiter assembly are suitable to dilute the samples before transferring them to the testwells: Order No 73.1055, Sarstedt, Postfach 1220, 51582 Nümbrecht, Germany.

The following reagent reservoirs are suitable to suck up reagents by a multichannel pipette: Order No 7782401, ICN Pharmaceuticals, Mühlgrabenstr. 12, 53334 Meckenheim, Germany.

PRINCIPLE OF THE ASSAY

The Anti-MPO (P-ANCA) ELISA is a sandwich enzyme immunoassay. During sample incubation anti-MPO antibodies contained in standards and patient sera bind to the highly purified myeloperoxidase, which is fixed on the surface of microtiter wells. After a washing step, peroxidase-conjugated anti-human-IgG is added, which binds to the autoantibodies. After a second washing step, the amount of fixed enzyme is determined by the oxidation of tetramethylbenzidine (TMB) to a blue coloured product. The addition of sulphuric acid stops this indicator reaction and causes a colour shift to yellow. The optical density of the samples is read in a photometer at 450 nm (reference wavelength 570-650 nm) and the concentrations are calculated by means of the parallel processed standards. Because a WHO standard does not exist for these autoantibodies an arbitrary definition was chosen in 1990.

The assay can also be used for screening, when only the standards S0 (diluent for samples) and S5 (5 U/ml Anti-MPO) are employed. These standards are used to determine the cut-off values (see INTERPRETATION OF RESULTS).

REAGENTS PROVIDED IN THE KIT (for *in vitro* use only)

MPO : 12 microtiter strips with 8 breakable wells each, coated with highly purified myeloperoxidase
dil : 2 bottles (60 ml each) diluent for samples (contains phenol)
S5 : 1 vial (0.75 ml) human standard, 5 U/ml Anti-MPO (contains phenol)
S20 : 1 vial (0.75 ml) human standard, 20 U/ml Anti-MPO (contains phenol)
S50 : 1 vial (0.75 ml) human standard, 50 U/ml Anti-MPO (contains phenol)
S100 : 1 vial (0.75 ml) human standard, 100 U/ml Anti-MPO (contains phenol)
ø : 1 vial (0.75 ml) human negative control (contains phenol)
+ : 1 vial (0.75 ml) human positive control, 60 U/ml Anti-MPO (contains phenol)
wash : 1 bottle (50 ml) washing buffer, 20x concentrate
conj : 1 vial (12 ml) anti-human-IgG/POD-conjugate (contains phenol)
subs : 1 vial (1.0 ml) TMB substrate reagent, 20x concentrate
stop : 1 bottle (30 ml) stopping solution (0.5 M sulphuric acid)
Additionally, the testkit contains 2 self-adhesive foils. A stripejector is available on request.

STORAGE AND STABILITY

The kit must be stored at 2° to 8°C and is stable up to the imprinted expiration date. All reagents must be brought to room temperature prior to use and (except for the washing buffer) returned to storage conditions immediately after use. The washing buffer is stable at room temperature (even in working dilution) up to the expiration date of the kit.

The TMB substrate reagent is light-sensitive and should not be exposed to light longer than necessary.

PRECAUTIONS

Standards and controls contain human blood components. These have been tested and found nonreactive for hepatitis B_S-antigen and for anti-HIV antibodies. Nevertheless all blood derivatives are to be regarded as potentially infectious and must be handled with appropriate care and caution.

The stopping solution (diluted sulphuric acid), the TMB substrate reagent and the reagents which contain phenol must be handled with caution since they can cause skin irritation. Avoid swallowing and contact with the skin or mucous membranes. If these reagents come into contact with skin or mucous membranes, wash thoroughly with water.

PREPARATION OF REAGENTS

Microtiter strips: Keep the closed pouch at room temperature for minimum 10 minutes and cut it open near to the edge. The microtiter strips should be ejected from the frames using the stripejector, which is available on request, because the bottom side of the wells should not be touched. If necessary, a required number of wells can be broken apart from the strips. Take the strips that are not needed out of the frame, put them back into the pouch (leave desiccant in the pouch) and seal the zip lock between fingers starting from the edge, because this is the only way to close it safe. For the use of further testkits it is recommended to keep always one frame, in which the needed strips can be placed. Press the strips and separated wells firmly into the frame to ensure that they do not fall out during the washing procedure. Especially, if an 8-channel washing device is employed, some used wells should be washed and stored for putting them into empty positions of one column during later runs of the assay.

Washing buffer: Dilute the content of the bottle washing buffer 20x concentrate (50 ml) with deionized water up to 1000 ml. Possibly existing crystalline deposit in the concentrate is concentration-conditioned. It must be transferred into the measuring vessel and dissolved after adding the deionized water. Diluted washing buffer is stable up to the expiration date, even when stored at room temperature.

Substrate: The TMB substrate reagent (20x concentrate) has to be diluted 1:20 with distilled water shortly before it is needed. Only the required amount of working dilution should be prepared. For example, if 16 wells are used, a volume of 2 ml is sufficient (1.9 ml distilled water plus 0.1 ml TMB concentrate).

The TMB concentrate and the working dilution are light sensitive and must be protected from the light.

All the other reagents are ready for use.

PROCEDURAL NOTES

The Anti-MPO (P-ANCA) ELISA is prepared for parallel processing with the Anti-PR3 (C-ANCA) ELISA (Cat.-No.: EA004/96). For simultaneous determination of both autoantibody specificities, following reagents can be used from either of the kits: Sample diluent, negative control, washing buffer, conjugate, substrate and stopping solution. However, in the course of one test performance, conjugate or substrate of only one of the testkits should be used. If more reagent is needed than available in one kit, the required volume may be pooled in advance from the respective reagents of both kits.

Reagents that are specific for the Anti-MPO (P-ANCA) ELISA (microtiter strips, standards and positive control) have green marks, respectively green lettering of the cap.

PREPARATION OF SAMPLES

If the assay is performed within 5 days after blood collection, storage of the sera at 2˚ to 8˚C is sufficient; otherwise they should be stored at -20˚C or deeper. To avoid repeated freeze-thaw cycles the sera should be aliquoted.

Dilute patient sera 1:50 (490 µl diluent for samples + 10 µl serum).

If a serum contains more than 100 U/ml Anti-MPO, it is above the measuring range and has to be diluted further in steps of 1:20 (475 µl sample diluent + 25 µl predilution) until the OD-values are within the range of the calibration curve. In the producers experience sera generally do not exceed 10,000 U/ml and only about 5 % of all positive sera have values above 2000 U/ml. As a consequence 95 % of all positive sera are in the measuring range if 2 sample dilutions are tested (1:50 and 1:1000).

ASSAY PROCEDURE

Process volume is 100 µl.

1. Sample incubation: Fill into the microtiter wells (preferentially in duplicates)

ready to use calibration components	ready to use controls	diluted patient sera (samples)
S0 (= diluent for samples)	ø negative control	
S5	+ positive control	
S20		
S50		
S100		

   Cover with self-adhesive foil, and incubate for 2 hours at room temperature.
2. Washing: Aspirate the wells and perform 4 wash cycles. One cycle consists of filling each well with 300-350 µl washing buffer, incubation for approximately 30 seconds and aspiration of the wells. After the last cycle residual liquid must be removed by tapping the inverted plate on a paper towel. This should also be done, if an automatic washing device is used.
3. Conjugate incubation: Fill 100 µl undiluted conjugate into each well, cover with self-adhesive foil and incubate for 1 hour at room temperature.
4. Washing: Wash again as described above (2.).
5. Substrate reaction: Fill 100 µl freshly prepared substrate working dilution into each well and incubate in the dark for 15 minutes at room temperature.
6. Stopping of substrate reaction: Add 100 µl stopping solution into each well in the same order and time cycle as done with the substrate working dilution.

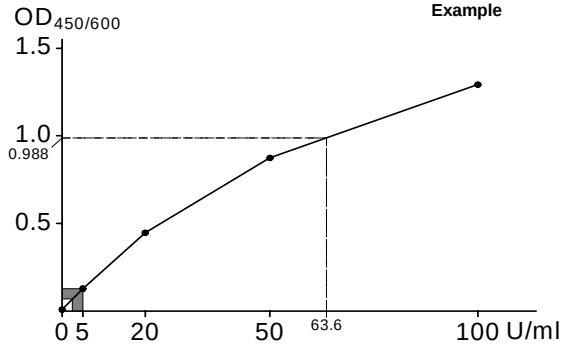
READING

Read the absorbance of wells immediately after stopping at 450 nm in a microtiter plate reader versus air, using a reference wavelength between 570 and 650 nm. If zero blanking of the photometer with liquid is necessary, 200 µl/well of stopping solution can be utilized for this purpose. If the optical density could only be measured later than 10 minutes after stopping, the plate should be covered with self-adhesive foil and stored in the dark. At room temperature storage is possible for up to 2 hours and at 2˚ to 8˚C for up to 24 hours. In the latter case reading should only be started after condensing water is dried from the bottom of the wells.

CALCULATION OF RESULTS

Calculate the concentration values (U/ml) of the samples by means of graph paper or a computer program utilizing linear interpolation (polygonal data handling) or an appropriate curve fitting. If a sample has been diluted further than 1:50, the concentration read from the calibration curve must be multiplied by the further dilution factor.

Example



component	$OD_{450/600}$
S0 (diluent for samples)	0.009
S5	0.128
S20	0.447
S50	0.874
S100	1.292
negative control	0.021
positive control	0.988

By linear interpolation from the calibration curve, the Anti-MPO concentration of the positive control is determined to be 63.6 U/ml.