Scheme of the assay

samples (1:50), standards and controls (ready to use) 100 µl

incubation: 2 hours

washington (4x) ↓

conjugate (ready to use) 100 µl

incubation: 1 hour

washington (4x) ↓

substrate (1:20) 100 µl

incubation: 15 minutes

stopping solution (ready to use) 100 µl ↓

reading of absorbance at 450/600 nm

Operating instructions

Anti-PR3 (C-ANCA) ELISA

Microtiter plate enzyme immunoassay for the detection and quantification of anti-proteinase 3 autoantibodies in serum

(for in vitro use only)

Cat. No. : EA004/96
Determinations : 96
Storage : 2° - 8°C

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INTRODUCTION
Since 1985, detection of anti-neutrophil cytoplasm antibodies (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).

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 autoantibodies (e.g. in SLE). Therefore, specific detection of anti-MPO antibodies, as marker for Microscopic Polyangiitis (17), is only possible by means of an assay like the Anti-MPO (P-ANCA) ELISA, (Cat.-No.: EA005/96) where highly purified myeloperoxidase is used as antigen.

DIAGNOSTIC RELEVANCE
Anti-PR-3 antibodies (Anti-PR3) are marker for Wegener’s Granulomatosis (WG), but also occur in patients with Microscopic Polyangiitis (21). In a study (7), including 277 patients with WG and 1657 control patients, the specificity of the Anti-PR3 ELISA for WG was determined to be 98%. The sensitivity of the Anti-PR-3 ELISA depended on disease activity and extent. It was found to be 93% for patients with active generalized disease, 60% for patients with active lobar disease and 40% for patients in remission. Follow-up observations demonstrated that autoantibody changes paralleled changes in disease activity and helped to distinguish relapses of WG from other intercurrent illnesses, mainly infections, which are always a threat for patients on immunosuppressive treatment (7). In a 16 month prospective study (10) on 35 patients with WG sevenent relapses were observed which were all preceded (by a mean period of 7 weeks) by a significant rise in C-ANCA-titer. The findings were reproduced (24). Additionally, this group has shown that relapses in WG can be prevented by early treatment based on significant rises of C-ANCA-titer (11).

REFERENCES
INTERPRETATION OF RESULTS

Samples with values lower than 2.5 U/ml are considered negative for Anti-PR3. 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-PR3.

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-PR3 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-PR3 (C-ANCA) ELISA is a sandwich enzyme immunoassay. During sample incubation anti-PR-3 antibodies contained in standards and patient sera bind to the highly purified proteinase 3, 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 1987.

The assay can also be used for screening, when only the standards S0 (diluent for samples) and S5 (5 U/ml Anti-PR3) 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)

PR3 : 12 microtiter strips with 8 breakable wells each, coated with highly purified proteinase 3
dil : 2 bottles (60 ml each) diluent with highly purified proteinase 3
S5 : 1 vial (0.75 ml) human standard, 5 U/ml Anti-PR3 (contains phenol)
S20 : 1 vial (0.75 ml) human standard, 20 U/ml Anti-PR3 (contains phenol)
S50 : 1 vial (0.75 ml) human standard, 50 U/ml Anti-PR3 (contains phenol)
S100 : 1 vial (0.75 ml) human standard, 100 U/ml Anti-PR3 (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-PR3 (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 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 stripjector, 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 safely. 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-PR3 (C-ANCA) ELISA is prepared for parallel processing with the Anti-MPO (P-ANCA) ELISA (Cat.-No.: EA005/96). 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\(^{\circ}\) to 8\(^{\circ}\)C is sufficient; otherwise they should be stored at -20\(^{\circ}\)C or deeper. To avoid repeated freeze-thaw cycles the sera should be aliquoted.

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

If a serum contains more than 100 U/ml Anti-PR3, it is above the measuring range and has to be diluted further in steps of 1:20 (475 \(\mu\)l sample diluent + 25 \(\mu\)l 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 \(\mu\)l.

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

<table>
<thead>
<tr>
<th>ready to use calibration components</th>
<th>ready to use controls</th>
<th>diluted patient sera (samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0 ( = diluent for samples)</td>
<td>e negative control</td>
<td>+ positive control</td>
</tr>
<tr>
<td>S5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 \(\mu\)l 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 \(\mu\)l 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 \(\mu\)l 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 \(\mu\)l stopping solution into each well and incubate in the dark for 15 minutes and same 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 \(\mu\)l/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\(^{\circ}\) to 6\(^{\circ}\)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.

<table>
<thead>
<tr>
<th>component</th>
<th>OD(_{450/600})</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0 (diluent for samples)</td>
<td>0.007</td>
</tr>
<tr>
<td>S5</td>
<td>0.142</td>
</tr>
<tr>
<td>S20</td>
<td>0.532</td>
</tr>
<tr>
<td>S50</td>
<td>1.075</td>
</tr>
<tr>
<td>S100</td>
<td>1.589</td>
</tr>
<tr>
<td>negative control</td>
<td>0.016</td>
</tr>
<tr>
<td>positive control</td>
<td>1.210</td>
</tr>
</tbody>
</table>

By linear interpolation from the calibration curve, the Anti-PR3 concentration of the positive control is determined to be 63.1 U/ml.