ELISA

for the determination of autoantibodies against

Myeloperoxidase (p-ANCA)

Directions for use

REF 2211FE00.FWD ∑ 12 x 8 determinations

IVD CE

8°C

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The product described herein has been manufactured in compliance with IVD directive 98/79/EG.

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1. Introduction and background

Anti-neutrophil cytoplasmic antibodies (ANCA), originally identified by immunofluorescence assays (IFA), are directed against cytoplasmic components of neutrophil granulocytes and monocytes. They have proven to be a useful serologic marker for a number of systemic, autoimmune mediated vasculitides (1, 2).

A perinuclear (p-ANCA) staining pattern of the neutrophil substrate is indicative for autoantibodies against myeloperoxidase (MPO), a 145 kDa large, cationic, covalently linked alpha_2-beta_2 complex. MPO autoantibodies are most closely associated with one form or another of microscopic polyangiitis. They also occur in patients with polyangiitis overlap syndrome without renal involvement, idiopathic or vasculitis-associated crescentic glomerulonephritis, classic polyarteritis nodosa and Churg-Strauss syndrome (3). The titer of MPO antibodies is considered to reflect flares and remissions of these diseases in individual patients (4).

The present enzyme-linked immunosorbent assay (ELISA) is intended for the quantitative or qualitative determination of IgG antibodies in human serum, directed against MPO. The immobilised antigen is a highly purified preparation of MPO, isolated from human granulocytes. The test is fast (incubation time 30/30/30 minutes) and flexible (divisible solid phase, ready-to-use reagents). Six calibrators allow quantitative measurements; a negative and a positive control check the assay performance.

2. Warnings and precautions

The test kit is intended for **in vitro** diagnostic use only; not for internal or external use in humans or animals.

Do not use reagents beyond their expiration dates.

Adherence to the protocol is strongly recommended.

The sample buffer, calibrators and controls contain Na-azide as preservative. The wash buffer contains bromonitrooxane and the conjugate methylisothiazolone / bromonitrooxide as preservative. The substrate contains 3, 3', 5, 5'-tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2). The stop solution, 0.2 M sulfuric acid (H_2SO_4), is acidic and corrosive.
The above mentioned reagents may be toxic if ingested. Follow routine precautions for handling hazardous chemicals. Avoid all body contact, wear gloves and eye protection. If one of the reagents comes into contact with skin or mucous membrane, wash thoroughly with water. Never pipette by mouth. Dispose in a manner complying with local/national regulations.

Na-Azide may react with lead and copper plumbing to form explosive metal azides. On disposal, flush with a large amount of water to prevent azide build-up.

The calibrators and controls contain components of human origin. They have produced negative results when tested for Human Immunodeficiency Virus (HIV)-Ag, hepatitis B surface (HBs)-Ag, HIV 1/2-Ab and hepatitis C Virus (HCV)-Ab, in FDA-approved or European Directive 98/79/EG-compliant tests. However, no known test can guarantee that products derived from human blood will not be infectious. They should therefore be handled as if capable of transmitting infectious agents, and discarded appropriately. Please refer to CDC (Center of Disease Control, Atlanta, USA) or other local/national guidelines on laboratory safety and decontamination procedures.

3. Principle of the test

The wells of the solid phase are coated with MPO. On this surface, the following immunological reactions take place:

1st reaction: MPO-specific antibodies present in the sample bind to the immobilised antigen, forming the antigen-antibody complex. Then, non-bound sample components are washed away from the solid phase.

2nd reaction: A second antibody, directed at human IgG antibodies and conjugated with horse-radish peroxidase (HRP), is added. This conjugate binds to the complex. Then, excess conjugate is washed away from the solid phase.

3rd reaction: The enzyme-labelled complex converts a colourless substrate into a blue product. The degree of colour development reflects the concentration of MPO antibodies (IgG) in the sample.
4. Contents of the kit

a. 1 microwell plate, coated with MPO and hermetically packed in a foil laminate pouch together with a desiccant bag. The plate consists of 12 strips, each of which can be broken into 8 individual wells.

\[
\text{MWP 12x8}
\]

b. Sample buffer, 100 mL, ready-to-use, orange coloured. Contains Tris-buffered saline (TBS), bovine serum albumin (BSA), Tween and Na-azide.

\[
\text{BUF SPL}
\]

c. Wash buffer, 100 mL, 10x-concentrate, blue coloured. Contains TBS, Tween and bromonitrodioxane.

\[
\text{BUF WASH 10x}
\]

d. 6 calibrators, 2,0 mL each, 0 - 3,0 - 8,0 - 18 - 45 and 100 U MPO antibodies (IgG) / mL, ready-to-use, gradually blue coloured. Contain TBS, BSA, Tween and Na-azide.

\[
\text{CAL 1-6}
\]

e. Negative and positive control, 2,0 mL each, ready-to-use, green and red coloured, respectively. Contain TBS, BSA, Tween and Na-azide.

\[
\text{CONTROL - CONTROL +}
\]

f. Anti-human IgG HRP conjugate, 14 mL, ready-to-use, red coloured. Buffered solution containing stabilising protein, methylisothiazolone and bromonitrodioxane.

\[
\text{CONJ IgG}
\]

g. Substrate solution, 14 mL, ready-to-use, colourless. Contains a buffered solution of TMB and H$_2$O$_2$. Contained in a vial impermeable to light.

\[
\text{SUBS TMB}
\]
h. Stop solution (0.2 M H₂SO₄), 14 mL, colourless, ready-to-use. Caution: sulfuric acid is corrosive.

SOLN STOP

i. Directions for use

j. Lot-specific certificate of analysis

5. Materials required but not supplied

a. Deionised or distilled water

b. Graduated cylinder, 1000 mL

c. Tubes for sample dilution (transfer tubes in the microwell plate format recommended)

d. Pipettes for 10, 100 and 1000 µL (1- and 8-channel pipettes recommended)

e. Microwell plate washer (optional)

f. Microwell plate photometer fitted with a 450 nm filter

g. ELISA evaluation program (recommended)

6. Storage of the kit

Store kit at 2 - 8°C. It is stable up to the expiry date stated on the label of the box. Do not use kit beyond its expiry date.

7. Reagent and sample preparation / specimen requirements

Do not exchange or pool corresponding components from different kits, due to possibly different shipping or storage conditions.
a. Before opening the pouch of the solid phase, it must have reached room temperature. Remove the supernumerary microwells from the frame and immediately put them back into the pouch, together with the desiccant bag. Reseal the pouch hermetically and keep it refrigerated for future use.

b. Dilute the wash buffer 10x-concentrate (100 mL, blue) with 900 mL deionised water. Mix thoroughly. The diluted buffer is stable for several weeks if stored refrigerated (2 - 8°C).

c. Preparation of the samples: Handle patient specimens as if capable of transmitting infectious agents. Prepare sera using normal laboratory techniques and dilute them 1/100, e.g. 10 µL serum + 990 µL sample buffer. Mix thoroughly.

For rapid dispensing during the assay procedure, preparation of the calibrators, controls and samples in microwell transfer tubes is recommended. This allows the operation of an 8-channel pipette during the assay procedure.

If samples are not assayed immediately, they should be stored at 2 - 8°C and assayed within 3 days. For longer storage, -20°C or lower temperature are recommended. Repeated freezing and thawing of sera should be avoided. Thawed samples must be mixed prior to diluting.

Specimen requirements: Highly lipemic, haemolysed or microbially contaminated sera may cause erroneous results and should be avoided.

8. Assay procedure

8.1. Manual operation
Before starting the assay, all components of the kit must have reached room temperature (23 ± 3°C).

To achieve best results, i.e. the maximum ratio between specific and background signal, careful washing is essential (steps a, c and e). It is crucially important to remove the wash solution completely. For that purpose, tap the plate firmly on several layers of absorbent tissue. Automated washers must be verified according to results obtained by manual washing.

a. Immediately prior to use, wash the solid phase once: fill wells with 350 µL wash buffer each, soak for about 10 seconds in the wells and remove.
b. Dispense the calibrators (2.0 mL each, ready-to-use, gradually blue), controls (2.0 mL each, ready-to-use, green and red) and the diluted samples rapidly into the microwells; 100 µL per well. Duplicate measurements are recommended.

Incubate the plate for 30 minutes at room temperature (23 ± 3°C).

c. Wash the wells 4 times as in step a.

d. Rapidly (preferably using an 8-channel pipette) dispense the conjugate (14 mL, ready-to-use, red); 100 µL per well. Incubate the plate as in step b.

e. Repeat wash step c.

f. Rapidly (preferably using an 8-channel pipette) dispense the substrate solution (14 mL, ready-to-use, colourless, black vial); 100 µL per well. Incubate the plate as in step b. As the substrate is photosensitive, avoid intense light exposure (e.g. direct sunlight) during incubation.

g. Rapidly (preferably using an 8-channel pipette) dispense the stop solution (14 mL, ready-to-use, colourless. Caution: corrosive!); 100 µL per well. Use the same sequence as for the substrate. The colour changes from blue to yellow. Agitate the plate, preferably on an orbital shaker, for about 10 seconds.

h. Immediately read the absorbance in the microwell plate photometer at 450 nm.

Store the remainder of the reagents refrigerated (2 - 8°C) if they are to be used again.

8.2. Dynex DS2 automated ELISA system
This product has been validated for use with the Dynex DS2 automated ELISA system. A suitable program file for assay execution and evaluation is available on request. The parameters of this program are merely a proposal and may need to be adapted by the operator to the requirements of the actual assay. In general terms, we have attempted to stick as close as possible to the protocol of manual operation, as above. However, due to the necessarily elevated temperature within the DS2, the substrate incubation period had to be shortened. Article 11.8. gives a performance comparison between manual assay operation and the DS2 ELISA system.
9. Evaluation and quality control

Quantitative evaluation: The data obtained are quantitatively evaluated with the standard curve, as shown below. However, the depicted curve can only serve as a model. It can not substitute the measurement of the calibrators, together with the controls and actual samples. The curve has been constructed with a conventional ELISA evaluation program, using a 4-parameter function. The Spline approximation is also appropriate.

If no computer-supported evaluation is possible, the standard curve may be drawn by hand. It allows transformation of the absorbance value of a sample into its concentration, i.e. into U MPO antibodies (IgG) per mL serum.

Qualitative evaluation: The test may also be evaluated in a qualitative manner. This requires measurement of the positive control only. Nevertheless, measurement and examination of the negative control is recommended (see below: quality control).

In qualitative test evaluation, the absorbance of the samples is compared with the borderline absorbance (= cut-off). It is determined according to the following formula:

\[
\text{absorbance}_{\text{borderline}} = \text{absorbance}_{\text{positive control}} \times \text{factor}
\]
The factor depends on the kit lot and is quoted in the lot-specific certificate of analysis which is included with each test kit. Example:

\[
\begin{align*}
\text{absorbance}_{\text{positive control factor}} &= 1250 \text{ mOD} \\
\text{absorbance}_{\text{borderline}} &= 0,35 \\
\text{absorbance}_{\text{sample}} &= 1250 \text{ mOD} \times 0,35 = 438 \text{ mOD}
\end{align*}
\]

In order to gain an impression of how positive a particular sample is for MPO-Ab (IgG), one may calculate the ratio, according to the formula:

\[
\text{ratio} = \frac{\text{absorbance}_{\text{sample}}}{\text{absorbance}_{\text{borderline}}}
\]

Example:

\[
\begin{align*}
\text{absorbance}_{\text{sample}} &= 1480 \text{ mOD} \\
\text{absorbance}_{\text{borderline}} &= 438 \text{ mOD} \\
\text{ratio} &= \frac{1480 \text{ mOD}}{438 \text{ mOD}} = 3,4
\end{align*}
\]

Quality control: The positive and negative control check the assay performance. Their authorised values and acceptable ranges, respectively, are quoted in the lot-specific certificate of analysis. Values of the controls have to fall within the indicated ranges; otherwise, the results of the assay are invalidated.

10. Interpretation of results / limitations of the procedure

Based on the measurement of a blood donor and a positive collective of sera (see below), we suggest for the assessment of patient sera:

<table>
<thead>
<tr>
<th>quantitative evaluation</th>
<th>qualitative evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>U MPO-Ab (IgG) per mL serum</td>
<td>ratio</td>
</tr>
<tr>
<td>normal (negative) range</td>
<td>&lt; 9,6</td>
</tr>
<tr>
<td>cut-off</td>
<td>11,0</td>
</tr>
<tr>
<td>equivocal range</td>
<td>9,6 - 12,7</td>
</tr>
<tr>
<td>positive range</td>
<td>&gt; 12,7</td>
</tr>
</tbody>
</table>

These specifications are given as an indication only; in order to check their accuracy, each analysis should include parallel samples of normal sera.
A negative test result indicates that the patient does not have an elevated level of IgG antibodies to MPO. It does not preclude the presence of autoantibodies against other neutrophilic antigens (e.g. elastase, cathepsin G) which can be responsible for perinuclear staining pattern in IFA analysis.

As antibodies to MPO are rarely found in normal individuals, a positive result should be considered as an indication for one of the vascular disorders mentioned in the beginning.

Specimens exhibiting results between the borderlines quoted above should be considered as equivocal and reported as such. It is recommended that a second sample be collected two weeks later and run in parallel with the first sample to document a possible change of antibody titer.

As with any serological test, the results should be interpreted in the light of the patient's symptoms and other diagnostic criteria.

11. Performance characteristics

11.1. Standardisation
The test is standardised with a purified serum preparation containing IgG antibodies specifically directed at MPO. This preparation is calibrated against a set of gradually positive sera, solely reserved for this purpose. The degree of sample reactivity is measured in arbitrary units (U/mL) since no international standard is available.

11.2. Analytical specificity
The test permits the specific determination of human IgG antibodies directed against MPO.

11.3. Detection limit (analytical sensitivity)
The detection limit is defined as that concentration of analyte that corresponds to the mean absorbance of sample buffer plus 3-fold standard deviation (s). It was determined as < 1 U MPO-Ab (IgG) per mL serum \( (n = 24) \). Recommended measuring range: 1 - 100 U MPO-Ab (IgG) per mL serum

11.4. Homogeneity of the solid phase
Measurement of the solid phase homogeneity is regular QC part of each production lot. This is determined by 288-fold measurement of a IgG-positive but non-saturating sample on 3 selected plates. Acceptance criterion: mOD-coefficient of variation (cv) over the plates < 8%. The figure below shows a representative excerpt (solid phase lot no. 0901C) of such an analysis.
<table>
<thead>
<tr>
<th>plate</th>
<th>early (n/10)</th>
<th>late (9n/10)</th>
<th>mean</th>
<th>Cv%</th>
</tr>
</thead>
<tbody>
<tr>
<td>row</td>
<td>1 2 6 7 11 12</td>
<td>1 2 6 7 11 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>line a</td>
<td>1194 1212 1222 1216 1212 1202</td>
<td>1216 1228 1238 1237 1264 1255</td>
<td>1225</td>
<td>1,7</td>
</tr>
<tr>
<td>line b</td>
<td>1205 1219 1219 1201 1198 1205</td>
<td>1207 1211 1196 1220 1239 1227</td>
<td>1212</td>
<td>1,1</td>
</tr>
<tr>
<td>line c</td>
<td>1184 1177 1192 1176 1174 1174</td>
<td>1170 1167 1183 1205 1195 1239</td>
<td>1186</td>
<td>1,7</td>
</tr>
<tr>
<td>line d</td>
<td>1191 1177 1193 1175 1181 1189</td>
<td>1188 1170 1187 1199 1201 1238</td>
<td>1191</td>
<td>1,5</td>
</tr>
<tr>
<td>line e</td>
<td>1175 1193 1189 1183 1175 1181</td>
<td>1163 1168 1171 1192 1200 1216</td>
<td>1184</td>
<td>1,3</td>
</tr>
<tr>
<td>line f</td>
<td>1188 1184 1190 1182 1185 1189</td>
<td>1164 1147 1160 1194 1210 1220</td>
<td>1184</td>
<td>1,7</td>
</tr>
<tr>
<td>line g</td>
<td>1200 1206 1220 1208 1193 1205</td>
<td>1181 1181 1177 1163 1200 1218</td>
<td>1196</td>
<td>1,5</td>
</tr>
<tr>
<td>line h</td>
<td>1208 1191 1209 1190 1194 1208</td>
<td>1182 1150 1165 1170 1212 1208</td>
<td>1191</td>
<td>1,7</td>
</tr>
<tr>
<td>mean</td>
<td>1193 1195 1204 1191 1189 1194</td>
<td>1184 1178 1185 1198 1215 1228</td>
<td><strong>1196</strong></td>
<td></td>
</tr>
<tr>
<td>cv%</td>
<td>0,9 1,3 1,2 1,3 1,1 1,1</td>
<td>1,6 2,4 2,1 2,0 2,0 1,3</td>
<td><strong>1,9</strong></td>
<td></td>
</tr>
</tbody>
</table>

![Graph of mOD 450nm](image1)

![Graph of mOD 650nm](image2)
11.5. Linearity
In order to assess the dose-response relationship of the test, positive sera were measured in serial 2-fold dilution. Acceptance criterion: linear regression of 4 successive dilutions must yield a correlation factor > 0.98. A typical result is depicted below.

![Linearity Diagram]

11.6. Precision
For the assessment of the test precision, the variability of results under the following conditions was determined: a. within 1 assay and between 3 assays, b. between 3 operators and c. between 2 kit lots.

a. Intra- and inter-assay variability (n = 24 and 72, respectively)

<table>
<thead>
<tr>
<th>sample</th>
<th>mean U/mL</th>
<th>variability (cv, %) intra-assay</th>
<th>inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8,2</td>
<td>4,6</td>
<td>4,7</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>4,4</td>
<td>4,6</td>
</tr>
<tr>
<td>3</td>
<td>46</td>
<td>3,0</td>
<td>3,2</td>
</tr>
</tbody>
</table>
b. Operator to operator variability (n = 12)

<table>
<thead>
<tr>
<th>sample</th>
<th>mean U/mL</th>
<th>variability (cv, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8,6</td>
<td>2,6</td>
</tr>
<tr>
<td>2</td>
<td>26</td>
<td>1,6</td>
</tr>
<tr>
<td>3</td>
<td>47</td>
<td>5,2</td>
</tr>
</tbody>
</table>

c. Variability between 2 kit lots (n = 6)

<table>
<thead>
<tr>
<th>sample</th>
<th>mean U/mL</th>
<th>variability (cv, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8,0</td>
<td>9,6</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>6,8</td>
</tr>
<tr>
<td>3</td>
<td>43</td>
<td>9,1</td>
</tr>
</tbody>
</table>

11.7. Frequency distribution of MPO-Ab (IgG)

This was analysed in a sera collective of 160 blood donors, equally distributed by sex and age, and a collective of 34 sera found positive for MPO autoantibodies according to a FDA-approved, CE-compliant reference ELISA. The following distribution of the analyte was observed:

<table>
<thead>
<tr>
<th>blood donor sera</th>
<th>positive sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>n: 160</td>
<td>n: 34</td>
</tr>
<tr>
<td>mean: 1,3 U/mL</td>
<td>mean: 132 U/mL</td>
</tr>
<tr>
<td>mean + s: 2,3 U/mL</td>
<td>mean - s: 29 U/mL</td>
</tr>
<tr>
<td>mean + 2s: 3,3 U/mL</td>
<td>mean - 2s: &lt; 0 U/mL</td>
</tr>
<tr>
<td>median: 1,1 U/mL</td>
<td>median: 102 U/mL</td>
</tr>
<tr>
<td>95th percentile: 2,1 U/mL</td>
<td>5th percentile: 27 U/mL</td>
</tr>
</tbody>
</table>

ROC-analysis of these data was used to determine the cut-off as 11,0 U/mL (5). The data presented here suggest a diagnostic specificity and sensitivity of the ELISA of 99 % and nearly 100 %, respectively. These values apply for the measured sera only; other collectives may yield different results.
positive sera

blood donor sera

U MPO-Ab/mL

relative frequency (%)

cut-off
11.8. Manual operation vs. Dynex DS2 automated ELISA system

Variability: Using specimen of one and the same kit lot, the variability of assay results were compared between manual operation and the Dynex DS2 automated ELISA system:

<table>
<thead>
<tr>
<th></th>
<th>manual operation</th>
<th>Dynex DS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>intra-assay variability</td>
<td>mean cv = 1,8 %</td>
<td>mean cv = 4,1 %</td>
</tr>
<tr>
<td>(n = 16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>inter-assay variability</td>
<td>mean cv = 2,8 %</td>
<td>mean cv = 4,6 %</td>
</tr>
<tr>
<td>(n = 48)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Standard curve: depicted in article 9

Correlation:

\[ y = 0.980 \times \quad r = 0.998 \]
12. Warranty

Steffens biotechnische Analysen GmbH (SBA) guarantees that the product delivered has been thoroughly tested to ensure that its properties specified herein are fulfilled. No further warranties are given.

The performance data presented here were obtained using the procedure indicated. Any modification in the procedure may affect the results in which case SBA disclaims all warranties whether expressed, implied or statutory. Moreover, SBA accepts no liability for any damage, whether direct, indirect or consequential, which results from inappropriate use or storage of the product.

13. Symbols

- **REF**: Article code
- **LOT**: Batch code
- **Σ**: Contains x determinations
- **IVD**: For *in vitro* diagnostic use
- **CE**: Conformité Européenne
- **Store shielded from sunlight**: Store shielded from sunlight
- **Store at 2 - 8°C**: Store at 2 - 8°C
Expiration date

Read “Directions for Use”

Warning

Biological risk

Manufactured by
14. References


15. Summary flow chart

a. Dilute the sera 1/100 in sample buffer (100 mL, ready-to-use, orange) and mix.

b. Dilute the wash buffer 10x-concentrate (100 mL, blue) with water and mix.

c. Wash the wells once with 350 µL wash buffer each. Dispense 100 µL of the calibrators (2,0 mL each, ready-to-use, gradually blue) and controls (2,0 mL each, ready-to-use, green and red) and of the diluted samples into the wells of the solid phase. Duplicate measurements are recommended. Incubate for 30 minutes at room temperature (23 ± 3°C).

d. Wash the wells 4 times with 350 µL wash buffer each.

e. Dispense 100 µL of the conjugate (14 mL, ready-to-use, red) into the wells. Incubate as in step c.

f. Repeat washing step d.

g. Dispense 100 µL of the substrate solution (14 mL, ready-to-use, black vial) per well. Incubate as in step c. Then, add 100 µL stop solution (14 mL, ready-to-use, colourless) per well and agitate the plate briefly.

h. Immediately measure the absorbance at 450 nm.

i. Quantitative evaluation: Determine the standard curve and, using this curve, transform the absorbance of the samples into their respective antibody concentration (U MPO-Ab (IgG)/mL).

j. Qualitative evaluation: Determine the borderline absorbance by multiplying the absorbance of the positive control with the factor shown in the certificate of analysis. Then, calculate the ratio of the samples by dividing their absorbance by the borderline absorbance.