ELISA

for the determination of antibodies (IgA) against a Modified Gliadin Peptide (MGP)

Directions for use

REF 0209HE00.FWD  12 x 8 determinations

IVD  

CE

2 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

Celiac disease (CD; synonyme: gluten-sensitive enteropathy) is caused by a hypersensitive reaction of genetically predisposed individuals to ingested gluten (1). Gluten is a set of proteins present in many kinds of cereal grain, e.g. wheat, oats, barley and rye. CD affects the upper small intestine: its morphological manifestation, the more or less complete atrophy of the villi, leads to malabsorption problems, e.g. chronic vitamin deficiency (2). However, the symptoms are variable or sometimes even absent (3).

It has been known for many years that elevated levels of gliadin-specific antibodies occur in the sera of celiac patients (4, 5, 6). Gliadin is a component of gluten and constitutes a predominant antigen. It is fragmented in the small intestine and the resulting peptides are deamidated by the enzyme tissue transglutaminase (tTG) which itself has been identified as the major CD autoantigen (7).

Recently, it has been shown that certain deamidated gliadin peptides are powerful immunogens and that antibodies directed at these peptides exhibit a better diagnostic accuracy for CD, as compared to antibodies directed at crude gliadin (8). At the same time, gliadin-specific antibodies (resp. antibodies against deamidated gliadin peptides) are considered more sensitive than tTG-directed autoantibodies when diagnosing CD in very young children (9).

The present enzyme-linked immuno sorbent assay (ELISA) is intended for the quantitative or qualitative determination of IgA antibodies in human serum, directed against a modified (deamidated) gliadin peptide (MGP). The immobilised antigen is a highly purified, synthetic peptide derivative. 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 bromonitrodioxane and the conjugate methylisothiazolone / bromonitrodioxane as preservative. The substrate contains 3, 3', 5, 5'-tetramethylbenzidine (TMB) and hydrogen peroxide (H2O2). The stop solution, 0,5 M sulfuric acid (H2SO4), 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, 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 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 MGP. On this surface, the following immunological reactions take place:

1st reaction: MGP-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 IgA antibodies and conjugated with horse-radish peroxidase (HRP), is added and 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 MGP antibodies (IgA) in the sample.
4. Contents of the kit

a. 1 microwell plate, coated with MGP 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.

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.

BUF SPL

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

BUF WASH 10x

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

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.

CONTROL -  CONTROL +

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

CONJ IgA

g. Substrate solution, 14 mL, ready-to-use, colourless. Contains a buffered solution of TMB and H2O2. Contained in a vial impermeable to light.

SUBS TMB
h. Stop solution (0.5 M H₂SO₄), 14 mL, colourless, ready-to-use. Caution: sulfuric acid is corrosive.

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, yellow); 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 MGP antibodies (IgA) 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:

\[
\text{factor} = 0.35 \\
\text{absorbance}_{\text{positive control}} = 1250 \text{ mOD} \\
\text{absorbance}_{\text{borderline}} = 1250 \text{ mOD} \times 0.35 = 438 \text{ mOD}
\]

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

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

Example:

\[
\begin{align*}
\text{absorbance}_{\text{borderline}} &= 438 \text{ mOD} \\
\text{absorbance}_{\text{sample}} &= 1480 \text{ mOD} \\
\text{ratio} &= \frac{1480 \text{ mOD}}{438 \text{ mOD}} = 3.4
\end{align*}
\]

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 U MGP-Ab (IgA) per mL serum</th>
<th>qualitative evaluation ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal (negative) range &lt; 6,7</td>
<td>&lt; 0.86</td>
</tr>
<tr>
<td>cut-off 8,0</td>
<td>1,00</td>
</tr>
<tr>
<td>equivocal range 6,7 – 9,6</td>
<td>0,86 - 1,17</td>
</tr>
<tr>
<td>positive range &gt; 9,6</td>
<td>&gt; 1,17</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 IgA antibodies to MGP. It does not preclude the possibility of an IgA deficiency. If clinical signs of CD are observed, IgG antibodies directed at MGP and/or tTG should be determined.

A positive result should be considered as an indication for CD. For confirmation, tTG antibodies (IgA) may be examined.

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 IgA antibodies specifically directed at MGP. 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 IgA antibodies directed against MGP.

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 MGP-Ab (IgA) per mL serum (n = 24). Recommended measuring range: 2 - 100 U MGP-Ab (IgA) 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 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. 2810H) 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>659 693 693 671 690 680</td>
<td>632 682 703 700 708 696</td>
<td>684</td>
<td>3,1</td>
</tr>
<tr>
<td>line b</td>
<td>691 686 677 679 689 662</td>
<td>690 686 699 699 720 673</td>
<td>688</td>
<td>2,1</td>
</tr>
<tr>
<td>line c</td>
<td>693 681 683 681 672 677</td>
<td>682 688 697 691 684 709</td>
<td>687</td>
<td>1,4</td>
</tr>
<tr>
<td>line d</td>
<td>690 689 690 690 684 698</td>
<td>686 696 706 707 715 712</td>
<td>697</td>
<td>1,5</td>
</tr>
<tr>
<td>line e</td>
<td>690 679 696 686 689 689</td>
<td>695 693 717 705 712 722</td>
<td>698</td>
<td>1,9</td>
</tr>
<tr>
<td>line f</td>
<td>691 686 699 701 698 695</td>
<td>698 697 714 706 703 715</td>
<td>700</td>
<td>1,2</td>
</tr>
<tr>
<td>line g</td>
<td>645 690 696 692 704 684</td>
<td>694 701 708 715 704 701</td>
<td>695</td>
<td>2,6</td>
</tr>
<tr>
<td>line h</td>
<td>648 664 689 705 693 675</td>
<td>699 706 718 728 724 710</td>
<td>697</td>
<td>3,5</td>
</tr>
<tr>
<td>mean</td>
<td>676 684 690 688 690 683</td>
<td>685 694 708 706 709 705</td>
<td>693</td>
<td></td>
</tr>
<tr>
<td>cv%</td>
<td>3,1 1,3 1,1 1,6 1,4 1,7</td>
<td>3,2 1,2 1,1 1,6 1,8 2,1</td>
<td>2,4</td>
<td></td>
</tr>
</tbody>
</table>
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.

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>13</td>
<td>2.2</td>
<td>2.7</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>4.2</td>
<td>6.3</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>2.3</td>
<td>3.3</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>13</td>
<td>4,6</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>3,5</td>
</tr>
<tr>
<td>3</td>
<td>44</td>
<td>1,7</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>12</td>
<td>5,3</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>10,4</td>
</tr>
<tr>
<td>3</td>
<td>44</td>
<td>3,5</td>
</tr>
</tbody>
</table>

11.7. Frequency distribution of MGP-Ab (IgA)

This was analysed in a sera collective of blood donors, equally distributed by sex and age, and a sera collective of CD patients, defined by biopsy and/or positive anti-tTG (IgA) result according to a 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: 400</td>
<td>n: 98</td>
</tr>
<tr>
<td>mean: 3,3 U/mL</td>
<td>mean: 69 U/mL</td>
</tr>
<tr>
<td>mean + s: 5,0 U/mL</td>
<td>mean - s: 10 U/mL</td>
</tr>
<tr>
<td>mean + 2s: 6,8 U/mL</td>
<td>mean - 2s: &lt; 0 U/mL</td>
</tr>
<tr>
<td>median: 2,8 U/mL</td>
<td>median: 53 U/mL</td>
</tr>
<tr>
<td>95th percentile: 6,5 U/mL</td>
<td>5th percentile: 3,6 U/mL</td>
</tr>
</tbody>
</table>

ROC-analysis of these data was used to determine the cut-off as 8,0 U/mL (10). Based on the data presented here, the diagnostic specificity and sensitivity of the ELISA was calculated to 97,5 and 84,7 %, respectively.
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 = 1.7 %</td>
<td>mean cv = 2.4 %</td>
</tr>
<tr>
<td>(n = 16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>inter-assay variability</td>
<td>mean cv = 3.7 %</td>
<td>mean cv = 4.7 %</td>
</tr>
<tr>
<td>(n = 48)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Standard curve: depicted in article 9

Correlation:

![Graph showing correlation between manual operation and Dynex DS2 results](image-url)
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
- Conformité Européenne
- Store shielded from sunlight
- 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, yellow) 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 (UMGP-Ab(IgA)/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.