Open tTG-ab ELISA (IgA)

ELISA for the determination of autoantibodies (IgA) against the open conformation of human Transglutaminase 2 (tTG) - Open tTG™

Directions for use

Art.-no. E006

For Research & Development Only
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1. Introduction and background

Autoantibodies against tissue transglutaminase (tTG, TG2) are a hallmark in celiac disease, a gluten sensitive enteropathy. In addition autoantibodies to tTG are found in sera of patients suffering from other glutensensitive disorders like dermatitis herpetiformis or glutensensitive ataxia.

A specific feature of the autoantigen tTG is its ability to adopt two different structural states: the closed conformation and the open conformation.

In healthy tissues or cells intracellular Ca\(^{2+}\)-concentrations are generally low, so that tTG is present in an inactive status characterized by a closed conformation as shown in fig.1.

![Fig.1.: inactive closed conformation (left) and active stabilized open conformation (right) of tissue transglutaminase. The grey area indicates regions of novel accessible epitopes.](image)

Increase of Ca\(^{2+}\)-concentration and substrate binding induces a tremendous shift of the beta-barrel domains resulting in the open conformation. The longitudinal open conformation renders novel epitopes accessible for antibody-binding (fig.1).
In inflamed tissues, where the Ca\(^{2+}\)-concentration is increased, the open conformation may be the dominant conformation of tTG.


**Open tTG™** - tTG in its open conformation - has been created by reacting recombinant human tTG (source: insect cells) with a novel irreversible inhibitor thus stabilizing the open conformation adopted by tTG upon substrate binding.

Zedira offers ELISA-kits for the detection of autoantibodies (IgA and IgG) to tissue-transglutaminase based on Open tTG™ as antigen.

Researchers of the University Tampere showed that in patients with diagnostic difficulties the Open tTG™-based ELISA is superior to the closed (standard) tTG version. They state that especially in patient’s sera with borderline or low conventional autoantibody titers despite active condition the Open tTG™-ELISA identified 93% of untreated celiac patients in contrast to 27% detected by conventional tTG-ELISA (Lindfors et al., J. Clin. Immunol. 2011 31:436-42. The authors conclude that the Open tTG™-ELISA constitutes a promising new non-invasive tool for the diagnostics and follow-up of celiac disease, especially in patients with diagnostic difficulties.

Researchers form Harvard Medical School, Celiac Center, used Open tTG™-ELISAs for testing dietary assessment of celiac patients. They conclude that Open tTG™-ELISAs may have higher sensitivity in the poor gluten free diet adherence group and higher specificity in the control population (Pallav et al., Dig Liver Dis. 2012;44(5):375-8).

Taken together scientific data suggest using Open tTG™ as antigen in tTG-autoantibody detection ELISAs.

The present ELISA is intended for the quantitative or qualitative determination of IgA antibodies in human serum, directed against the open conformation of tTG (Open tTG™).

The immobilised antigen in the present ELISA is a highly purified preparation of human recombinant tTG produced in insect cells and subsequently stabilized in its open conformation (Open tTG™). 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 use only. It is applicable only for research and development, not for diagnostic purposes.

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.

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 anti-HIV 1/2, anti-hepatitis C virus and hepatitis B surface antigen. 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.

3. Principle of the test

The wells of the solid phase are coated with tTG in open conformation.

1st reaction: tTG-specific antibodies present in the sample bind to the immobilised antigen, forming the antigen-antibody complex.

2nd reaction: A second antibody, directed at human IgA antibodies and labeled with horse-radish peroxidase (HRP), binds to the complex.

3rd reaction: The enzyme-labelled complex converts a substrate into a blue product. Samples containing IgA antibodies against tTG develop the blue colour, whereas samples without these antibodies remain colourless.
4. Contents of the kit

a. 1 microtitre plate, coated with purified tTG and packed in a resealable foil laminate pouch with a desiccant bag. The plate consists of 12 strips, each of which can be broken into 8 individual wells, thus providing maximum flexibility and economy in use of the assay.

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

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

d. 6 calibrators, 2.0 mL each, 0 – 1.0 – 3.0 - 10 - 30 and 100 U tTG antibodies (IgA) / mL, ready-to-use, gradually blue coloured. Contain TBS, BSA, Tween and Na-azide.

e. Negative and positive control, 2.0 mL each, ready-to-use, green and red coloured, respectively. Contain TBS, BSA, Tween and Na-azide.

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

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

h. 14 mL stop solution (0.5 M H2SO4), 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. Transfer tubes in the microplate format (recommended)
d. Pipettes for 10 and 100 µL (1- and 8-channel pipettes recommended)

e. Microplate washer (optional)

f. Microplate photometer fitted with a 450 nm and (optionally, as reference wavelength) a 620 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.

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. Take care to avoid any contamination of the buffer by serum.

For rapid dispensing during the assay procedure, preparation of the calibrators, controls and samples in microtitre 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

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), the negative and positive control (2.0 mL each, ready-to-use, green and red, respectively) and the diluted samples rapidly into the microwells; 100 µL per well. Duplicate measurements are recommended, especially for the calibrators and the controls.

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, 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); 100 µL per well. Use the same sequence as for the substrate. Agitate the plate, preferably on an orbital shaker, for about 10 seconds.

h. Immediately read the absorbance at 450 nm or preferably at 450 nm minus 620 nm.

Store the remainder of the reagents refrigerated if they are to be used again.

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 tTG antibodies (IgA) per mL serum.

Qualitative evaluation: The test may also be evaluated in a qualitative manner. This requires measurement of only the positive control. 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). The borderline absorbance 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:

\[
\begin{align*}
\text{absorbance}_{\text{positive control}} &= 1250 \, \text{mOD} \\
\text{factor} &= 0.35 \\
\text{absorbance}_{\text{borderline}} &= 1250 \, \text{mOD} \times 0.35 = 438 \, \text{mOD}
\end{align*}
\]

In order to gain an impression of how positive a particular sample is for tTG-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 sera collective (n = 160), equally distributed by sex and age, and of a collective of predefined positive sera (n = 80), we suggest for the assessment of unknown samples:

<table>
<thead>
<tr>
<th></th>
<th>quantitative evaluation</th>
<th>qualitative evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U tTG-Ab (IgA) per mL serum</td>
<td>ratio</td>
</tr>
<tr>
<td>normal (negative) range</td>
<td>&lt; 2.6</td>
<td>&lt; 0.90</td>
</tr>
<tr>
<td>cut-off</td>
<td>3.0</td>
<td>1.00</td>
</tr>
<tr>
<td>equivocal range</td>
<td>2.6 – 3.5</td>
<td>0.90 – 1.12</td>
</tr>
<tr>
<td>positive range</td>
<td>&gt; 3.5</td>
<td>&gt; 1.12</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.

11. Performance characteristics

Standardisation: The test is standardised with a purified serum preparation containing IgA antibodies specifically directed at tTG. 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.

Analytical specificity: The test permits the specific determination of human IgA antibodies directed against tTG (open conformation).

Recommended measuring range: 0.5 - 100 U tTG-Ab (IgA) per mL serum

Frequency distribution of Open tTG-Ab (IgA): In the two sera collectives mentioned above, the following distribution of the analyte was determined:

<table>
<thead>
<tr>
<th></th>
<th>blood donors (n = 160)</th>
<th>positive sera (n = 80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean</td>
<td>0.7 U / mL</td>
<td>64.2 U/mL</td>
</tr>
<tr>
<td>mean + 2s</td>
<td>1.5 U / mL</td>
<td>&lt; 0 U/mL</td>
</tr>
<tr>
<td>median</td>
<td>0.6 U / mL</td>
<td>42.8 U/mL</td>
</tr>
<tr>
<td>95% percentile</td>
<td>1.5 U / mL</td>
<td>6.3 U/mL</td>
</tr>
</tbody>
</table>
12. Warranty

Zedira 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 Zedira disclaims all warranties whether expressed, implied or statutory. Moreover, Zedira accepts no liability for any damage, whether direct, indirect or consequential, which results from inappropriate use or storage of the product.
13. 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 (or preferably at 450 nm minus 620 nm).

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