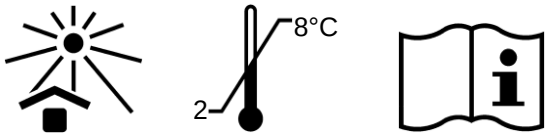
TG6-ab ELISA (IgA)

ELISA for the determination of autoantibodies (IgA) against human Transglutaminase 6

Directions for use

REF E103 12 x 8 determinations



For Research & Development Only

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Contents

Important Note

The TG6-ab ELISA (IgA) kit at hand with the Zedira product number E103 is the revised version of kit TG6-ab ELISA (IgA) with the product number E003.

Improvements:

1. No background subtraction is necessary anymore.

2. The calibrator has been adapted, so that the more relevant lower titers are covered. Titers (U/mL) obtained by the kit version E003 can roughly be calculated to titers of kit version E103 and vice versa:

   $titer_{E003} * 0{,}20 = titer_{E103}$

3. The cutoff has been redefined based on a sera collective of 38 blood donors, equally distributed by sex and age, and a collective of 86 patients with various neurological disorders.

The old kit version E003 is no longer available.

Document id./ver.-no.: E103 Manual V2.1/2022-07-25

1. Introduction and background

Gluten-related disorders generally are sub-grouped into celiac disease, wheat allergy and non-celiac gluten sensitivity, in total reaching a global prevalence of around 5%.

Celiac Disease (synonyms: celiac sprue, gluten-sensitive enteropathy) affects the upper small intestine. The most common chronic gastro-intestinal disorder is characterised by the inflammation of the small intestinal mucosa, leading to the more or less complete atrophy of the villi.

Autoantibodies to TG2 (tissue transglutaminase) and antibodies to DGP (deamidated gliadin peptides) are serological biomarkers for celiac disease.

TG2 belongs to a family of 8 human transglutaminase enzymes. Autoantibodies to TG3 (epidermal transglutaminase) are biomarker for dermatitis herpetiformis (Morbus Duhring-Brocq), a gluten related disorder of the skin, which may develop along with celiac disease.

Autoantibodies directed against neuronal transglutaminase (TG6) may occur in the sera of patients suffering from neurological disorders like e.g. ataxia, neuropathy, cerebral pulsy or stiffperson syndrome.

Autoantibodies to TG6 can be found in addition to TG2-autoantibodies in celiac patients, but were also found in TG2-autoantibody negative sera and in sera from patients without enteropathy (non-celiac patients). Therefore antibodies to TG6 may develop independent from celiac disease.

The present Enzyme-Linked Immuno Sorbent Assay (ELISA) is intended for the quantitative or qualitative determination of IgA antibodies in human serum, directed against TG6. The immobilised antigen is a highly purified preparation of human recombinant TG6. 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 (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, 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 TG6.

1st reaction: TG6-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 TG6 develop the blue colour, whereas samples without these antibodies remain colourless.

4. Contents of the kit

a. 1 TG6-coated microtitre plate packed in a resealable foil laminate pouch with a desiccant bag. The strips 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 - 3,0 – 8,0 - 18 - 45 and 100 U TG6 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 (0,2 M H_2SO_4), 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), 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 anti-h-IgA 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.

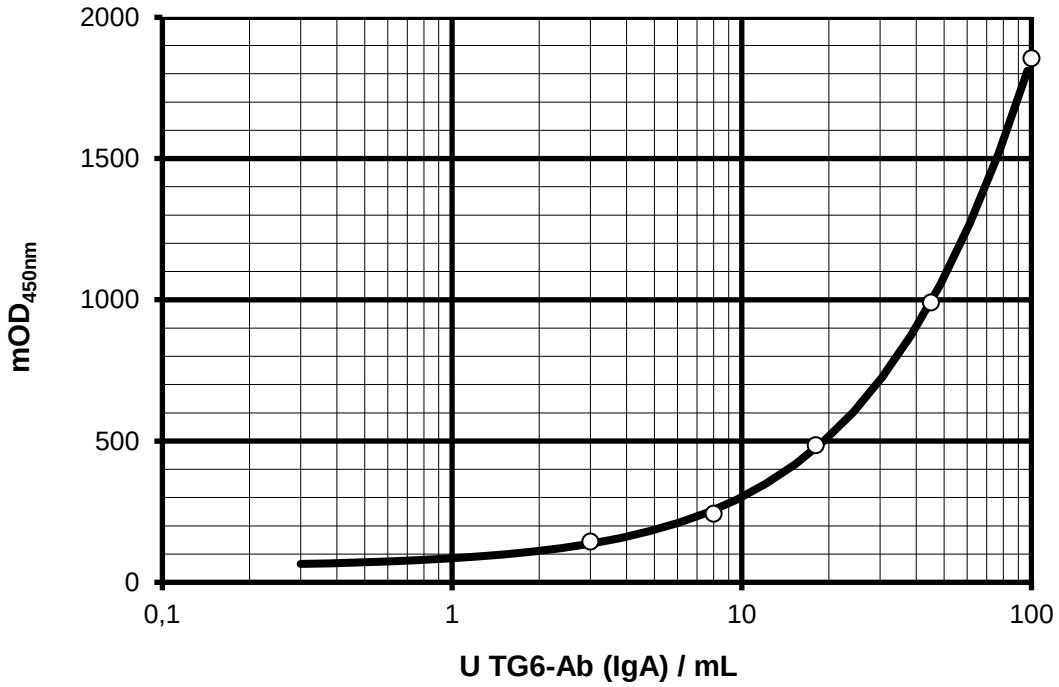
g. Rapidly (preferably using an 8-channel pipette) dispense the stop solution (14 mL, ready-to-use, colourless); 100 µL per well. Use the same sequence as for the substrate. Agitate the plate, preferably on an orbital shaker, for about 10 seconds.

h. Immediately read the absorbance in the microplate photometer 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 TG6 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:

$absorbance_{positive\ control}$	= 1250 mOD
factor	= 0,35
$absorbance_{borderline}$	= 1250 mOD x 0,35 = 438 mOD

In order to gain an impression of how positive a particular sample is for TG6-Ab (IgA), one may calculate the ratio, according to the formula:

$ratio = absorbance_{sample} / absorbance_{borderline}$

Example:

$absorbance_{borderline}$	= 438 mOD
$absorbance_{sample}$	= 1480 mOD
ratio	= 1480 mOD / 438 mOD = 3,4

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

The test in its current version is intended for research use only, not for diagnostic purposes. Based on the measurement of collectives of blood donor and positive sera (see below), we suggest for the assessment of serum samples:

	quantitative evaluation U TG6-Ab (IgA) per mL serum	qualitative evaluation ratio
normal (negative) range	< 2,6	< 0,89
cut-off	3,2	1,0
equivocal range	2,6 - 4,0	0,89 - 1,14
positive range	> 4,0	> 1,14

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 sample does not have an elevated level of IgA antibodies to TG6. It does not preclude the possibility of an IgA deficiency. If clinical signs are observed, IgG antibodies directed to TG6 and/or IgG/IgA antibodies to TG2 and/or gliadin (the main antigenic component of gluten) and/or DGP (deamidated gliadin peptide) could be determined.

A positive result could be considered as an indication for gluten-sensitive neurological disorders and/or Celiac Disease.

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

Standardisation: The test is calibrated with a purified preparation of TG6-specific antibodies conjugated with human IgA. 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 TG6.

Detection limit (analytical sensitivity): The detection limit is defined as that concentration of analyte that corresponds to the mean net absorbance of sample buffer plus 3-fold standard deviation (s). It was determined as < 0,5 U TG6-Ab (IgA) per mL serum (n = 24).

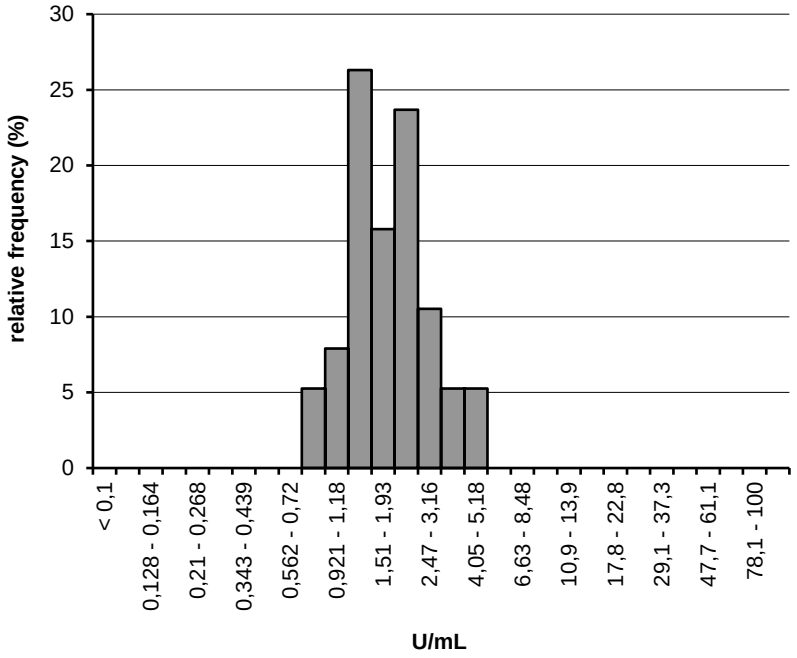
Recommended measuring range: 0,5 - 100 U TG6-Ab (IgA) per mL serum

Frequency distribution of TG6-Ab (IgA): This was analysed in a sera collective of 38 blood donors, equally distributed by sex and age, and a collective of 86 patients with various neurological disorders. The following distribution of the analyte was determined:

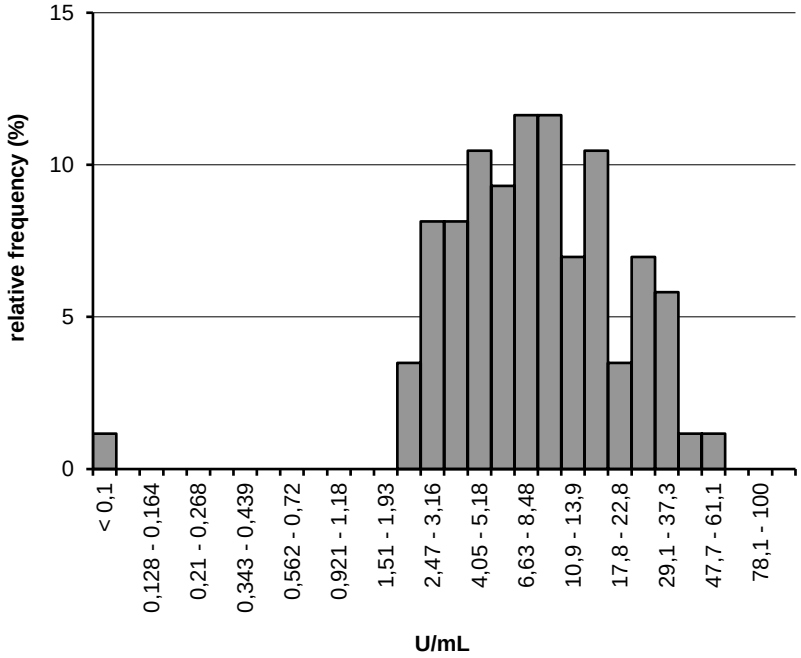
blood donor sera			intended positive sera		
n:	38		n:	86	
mean:	2,0	U/mL	mean:	11,6	U/mL
mean + s:	2,9	U/mL	mean - s:	1,5	U/mL
median:	1,8	U/mL	median:	8,1	U/mL
95^{th} percentile:	3,8	U/mL	5^{th} percentile:	2,5	U/mL

ROC-analysis of these data was used to determine the cut-off as 3,2 U/mL. The data presented here suggest a (theoretical) diagnostic specificity and sensitivity of the ELISA of about 89 % and 87 %, respectively. These values apply for the measured sera only; other collectives may yield different results.

Blood donor sera (n = 38)



Intended positive sera (n = 86)



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12. Warranty

Zedira GmbH 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. Arrange the suitable number of TG6-coated solid phase strips. 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 anti-h-IgA 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 (or 450 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 TG6-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.

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