Anti-Tissue-Transglutaminase IgA

ORG 540A

96 Tests

Immunometric Enzyme Immunoassay for the quantitative determination of IgA class autoantibodies to tTG

Instruction for use
WARNINGS AND PRECAUTIONS

All reagents of this test kit are strictly intended for in vitro use only. Please adhere strictly to the sequence of pipetting steps provided in this protocol. Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera.

All reagents should be stored refrigerated at 2 - 8 °C in their original container. Do not interchange kit components from different lots. The expiration dates stated on the labels of the shipping container and all vials have to be observed. Do not use kit components beyond their expiration dates.

Allow all kit components and specimen to reach room temperature prior to use and mix well. During handling of all kit reagents, controls and serum samples observe the existing legal regulations. The following precautions should be taken handling potentially infectious materials:
- do not eat, drink or smoke in areas where specimens or kit reagents are handled
- do not pipette by mouth
- wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.

The test kit contains components of human origin which, when tested by FDA-licensed methods, were found negative for hepatitis B surface antigen and for HIV antibody. No known test can guarantee, however, that products derived from human blood will not be infectious. Handle, therefore, all reagents and human blood derivatives, like plasma or serum samples, as if capable of transmitting infection.

Avoid contact with the TMB (3,3',5,5'-Tetramethyl-benzidine). If TMB comes into contact with skin wash thoroughly with water and soap.

The stop solution contains hydrochloric acid. If it comes into contact with skin, wash thoroughly with water and seek medical attention.

Avoid contact between the buffered Peroxide Solution and easily oxidized materials; extreme temperatures may initiate spontaneous combustion.

MATERIALS SUPPLIED

Package size 96 determ.
divisible microplate consisting of 12 modules of 8 wells, .................1
coated with human recombinant tTG
Anti-Tissue-Transglutaminase Calibrators in a serum/buffer matrix..... 6 vials, 1.5 ml each containing: 0; 5; 10; 25; 75 and 200 U/ml
Anti-Tissue-Transglutaminase Controls in a serum/buffer matrix ........ 2 vials, 1.5 ml each (positive and negative),.................................................................for the respective concentrations see the enclosed package insert
Anti-Tissue-Transglutaminase sample buffer, yellow, Concentrate ...... 1 vial, 20 ml
enzyme conjugate solution (light red), containing polyclonal rabbit ..... 1 vial, 15 ml
anti-h-IgA-IgG, labelled with horseradish peroxidase
TMB substrate solution..............................................................1 vial, 15 ml
stop solution (1 M hydrochloric acid)........................................1 vial, 15 ml
buffered wash solution, Concentrate ......................................1 vial, 20 ml
CONTROLS

A set of two controls is provided with the kit.

TECHNICAL DATA

Sample material: serum or plasma
Required sample volume: 10 µl of sample to be diluted 1:100 with sample buffer
100 µl pre-diluted sample per single determination
Total incubation time: 60 minutes at room temperature (20 - 28 °C)
Calibration range: 0 - 200 U/ml
Sensitivity: 1 U/ml
Storage: refrigerated at 2 - 8 °C
Shelf life: 12 months after manufacturing or until the expiration date printed on the labels
Package size: 96 tests

PRINCIPLE OF THE PROCEDURE

Anti-Tissue-Transglutaminase is an indirect solid phase enzyme immunometric assay (ELISA). It is designed for the quantitative measurement of IgA class autoantibodies directed against tTG. The assay is based on microplates coated with highly purified tTG. The microplate can be divided into 12 modules of 8 wells each or can be used complete for 96 determinations.

During this procedure the binding of present autoantibodies, as well as the formation of the sandwich complexes and enzymatic colour reaction take place during three different reaction phases:

**Phase 1:**
Calibrators, controls and pre-diluted patient samples are pipetted into the wells of the microplate. Any present antibodies bind to the immobilized antigens. After a 30 minutes incubation the microplate is washed with wash buffer for removing non-reactive serum components.

**Phase 2:**
An anti-human-IgA horseradish peroxidase conjugate solution is pipetted into the wells of the microplate to recognize the autoantibodies bound to the immobilized antigens. After a 15 minutes incubation any excessive enzyme conjugate, which is not specifically bound is washed away with wash buffer.

**Phase 3:**
A chromogenic substrate solution containing TMB (3,3‘,5,5’-Tetramethyl-benzidine) is dispensed into the wells. During 15 minutes of incubation the colour of the solutions change into blue. Colour development is stopped by adding 1 M hydrochloric acid as stop solution. The solutions colour change into yellow. The amount of colour is directly proportional to the concentration of IgA present in the original sample.

To read the optical density a microplate reader with a 450 nm filter is required. Bi-chromatic measurement with a 600-690 nm reference is recommended. The optical density for each calibrator may be graphically plotted against the concentration of IgA and unknowns extrapolated from the curve.
CLINICAL RELEVANCE

Tissue transglutaminase (t-TG, TT) has been reported to be the target for endomysial antibodies in celiac disease (CD).

Coeliac disease is a chronic disease affecting children as well as adults, with a genetic predisposition. The disease is elicited through the uptake of food containing gluten (e.g. bread made of wheat-flour, cakes, biscuits, noodles). The toxic agent is gliadin, the alcohol soluble fraction of wheat gluten. It's uptake leads to mucosal lesion of the small-intestine, due to a loss of absorptive villi leading to malabsorption of nutrients. As a result, the patient can show symptoms, like:

- Diarrhoea
- Slow growth
- Vomiting
- Tiredness
- Bad appetite

The risk of an intestinal lymphoma or any other gastrointestinal neoplasm is increased, if the disease remains untreated. Furthermore, longstanding untreated coeliac disease, even if clinically silent, predisposes for other autoimmune diseases. Therefore, population screening for IgA and IgG antibodies to tissue transglutaminase seems justified.

Coeliac disease, i.e. the flat mucosa can be reversed by a gluten-free diet, that needs to be strictly maintained.

The diagnosis of coeliac disease, according to The European Society of Paediatric Gastroenterology and Nutrition (ESPGAN) 1990, requires the identification of a single positive gut biopsy together with the demonstration of at least two of the three following IgA antibodies:

- IgG and IgA anti-gliadin antibodies
- IgA anti-endomysial antibodies
- Anti-reticulin antibodies

In 1997 it has been demonstrated that the enzyme tissue transglutaminase, being released from cells during inflammation, is the major if not sole target endomysial antigen.

Anti-tissue transglutaminase ELISA is an easy to use, non-invasive assay, that provides an efficient alternative to the immunofluorescent method and is ideal for screening of clinically suspected children or adults of coeliac disease.

NORMAL VALUES

In a normal range study with serum samples from healthy blood donors the following ranges have been established with the Anti-Tissue-Transglutaminase test:

Anti-Tissue-Transglutaminase IgA
Cut-Off: 10 U/ml

Positive results should be verified concerning the entire clinical status of the patient. Also every decision for therapy should be taken individually.

It is recommended that each laboratory establishes its own normal and pathological ranges. The values below should be regarded as guidelines only.
SPECIFICITY

The solid phase is coated with human recombinant tTG. Therefore the Anti-Tissue-Transglutaminase test kit recognises only autoantibodies specific for tTG.

CALIBRATION

Since no international reference preparations for Anti-Tissue-Transglutaminase autoantibodies is available, the assay system is calibrated in arbitrary units.

MATERIALS REQUIRED

Equipment
- Microplate reader capable for endpoint measurements at 450 nm
- Vortex mixer
- Pipettes for 10 µl, 100 µl and 1000 µl

Preparation of reagents
- distilled water
- graduated cylinder for 100 and 1000 ml
- plastic container for storage of the wash solution

Optional
- Multi-Channel Dispenser
- or repeatable pipet for 100 µl
- data reduction software

SPECIMEN COLLECTION AND PREPARATION

For determination of Anti-Tissue-Transglutaminase antibodies serum or plasma are the preferred sample matrixes.

All serum and plasma samples are prediluted 1 : 100 with sample buffer. Therefore 10 µl of sample may be diluted with 1000 µl of sample buffer.

The patients need not to be fasting, and no special preparations are necessary. Collect blood by venipuncture into vacutainers and separate serum or plasma from the cells by centrifugation after clot formation.

Samples may be stored refrigerated at 2 - 8 °C for at least 5 days. For longer storage of up to six months samples should be stored frozen at -20 °C. To avoid repeated thawing and freezing the samples should be aliquoted.

Neither Bilirubin nor Hemolysis have significant effect on the procedure.
PREPARATION AND STORAGE OF REAGENTS

All components of this test kit are supplied in a liquid format and ready to use, except the sample buffer and wash buffer. When stored refrigerated at 2 - 8 °C the components are stable for at least 30 days after opening or until the expiration date printed on the labels.

Remaining modules of the microplate should be stored refrigerated at 2 - 8 °C protected from moisture; store together with desiccant and carefully sealed in the plastic bag.

Preparation of sample buffer
Dilute the contents of each vial of the sample buffer concentrate (5x) with distilled water to a final volume of 100 ml prior to use. Store refrigerated: stable at 2 - 8 °C for at least 30 days after preparation or until the expiration date printed on the label.

Preparation of buffered wash solution
Dilute the contents of each vial of the buffered wash solution concentrate (50x) with distilled water to a final volume of 1000 ml prior to use. Store refrigerated: stable at 2 - 8 °C for at least 30 days after preparation or until the expiration date printed on the label.

NOTES ON TECHNIQUE

Control sera or pools should routinely be assayed as unknowns to check performance of the reagents and the assay.

For all controls, the respective concentrations are provided on the labels of each vial. Using these concentrations a calibration curve may be calculated to read off the patient results semi-quantitatively.

Pipetting and Sample Handling
Use a disposable-tip micropipette to dispense sera and plasma samples. Pipet directly to the bottom of the wells. To avoid carryover contamination change the tip between samples.

Patient samples expected to contain high concentrations should be additionally diluted with sample buffer before. Additional dilutions must be considered during calculation.
IMMUNOASSAY PROCEDURE

Do not interchange components of different lots.
All components should be at room temperature before use.
Dilute all patient samples 1:100 with sample buffer before assay. Therefore combine 10 µl of sample with 1000 µl of sample buffer in a polystyrene tube. Mix well. Calibrators and controls are ready to use and need not to be diluted.
1. Prepare a sufficient number of microplate modules to accommodate calibrators, controls and prediluted patient samples in duplicates.

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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tr>
<td>A</td>
<td>SA</td>
<td>SE</td>
<td>P1</td>
<td>P5</td>
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<tr>
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<td>SA</td>
<td>SE</td>
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<td>SF</td>
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<td>SD</td>
<td>C2</td>
<td>P4</td>
<td></td>
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</table>

SA - SF: standards A to F
P1, P2... patient sample 1, 2...
C1: positive control
C2: negative control

2. Pipet 100 µl of calibrators, controls and prediluted patient samples into the wells.
3. Incubate for 30 minutes at room temperature (20 - 28 °C).
4. Discard the contents of the microwells and wash 3 times with 300 µl of wash solution.
5. Dispense 100 µl of enzyme conjugate solution into each well.
6. Incubate for 15 minutes at room temperature.
7. Discard the contents of the microwells and wash 3 times with 300 µl of wash solution.
8. Dispense 100 µl of TMB substrate solution into each well.
9. Incubate for 15 minutes at room temperature.
10. Add 100 µl of stop solution to each well of the modules and leave untouched for 5 minutes.
11. Read the optical density at 450 nm and calculate the results. Bi-chromatic measurement with reference at 600-650 nm is recommended.

The developed colour is stable for at least 30 minutes.
Read optical densities during this time.

CALCULATION OF RESULTS

For the Anti-Tissue-Transglutaminase tests a 4-Parameter-Fit with lin-log coordinates for optical density and concentration is recommended. Smoothed Spline approximation and log-log coordinates are also suitable.

Recommended Lin-Log Plot
First calculate the averaged optical densities for each calibrator well. Use lin-log graph paper and plot the averaged optical density of each calibrator versus the concentration. Draw the best fitting curve approximating the path of all calibrator points. The calibrator points may also be connected with straight line segments. The concentration of unknowns may then be estimated from the calibration curve by interpolation.
ASSAY CHARACTERISTICS

Sensitivity
The lower detection limit for Anti-Tissue-Transglutaminase has been determined at 1 U/ml.

Parallelism
In dilution experiments sera with high antibody concentrations were diluted with sample buffer and assayed in the Anti-Tissue-Transglutaminase kit. The assay shows linearity over the full measuring range.

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<tbody>
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<td>IgA</td>
<td>1</td>
<td>1:200</td>
<td>102.1</td>
<td>105.1</td>
<td>97 %</td>
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<tr>
<td></td>
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<td>1:400</td>
<td>55.3</td>
<td>57.6</td>
<td>96 %</td>
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<tr>
<td></td>
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<td>1:800</td>
<td>27.7</td>
<td>28.8</td>
<td>96 %</td>
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<td>1:1600</td>
<td>14.9</td>
<td>14.4</td>
<td>103 %</td>
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<tr>
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<td>1:400</td>
<td>91.7</td>
<td>90.3</td>
<td>101 %</td>
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<tr>
<td></td>
<td></td>
<td>1:1600</td>
<td>21.8</td>
<td>22.6</td>
<td>97 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:3200</td>
<td>11.8</td>
<td>11.3</td>
<td>104 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:6400</td>
<td>6.1</td>
<td>5.7</td>
<td>110 %</td>
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Precision
Statistics were calculated for each of three samples from the results of 24 determinations in a single run for Intra-Assay precision and the run-to-run precision was calculated from the results of 5 different runs with 6 determinations each:

<table>
<thead>
<tr>
<th>Sample No</th>
<th>Intra-Assay</th>
<th>Inter-Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (U/ml)</td>
<td>CV (%)</td>
</tr>
<tr>
<td>1</td>
<td>35</td>
<td>4.8</td>
</tr>
<tr>
<td>2</td>
<td>87</td>
<td>5.4</td>
</tr>
<tr>
<td>3</td>
<td>155</td>
<td>6.1</td>
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</table>
REFERENCES


INCUBATION SCHEME

1. Pipet 100 µl calibrator, control or diluted patient sample. Incubate for 30 minutes at room temperature. Discard the contents of the wells and wash 3 times with 300 µl wash solution.

2. Pipet 100 µl enzyme conjugate. Incubate for 15 minutes at room temperature. Discard the contents of the wells and wash 3 times with 300 µl wash solution.

3. Pipet 100 µl substrate solution. Incubate for 15 minutes at room temperature.

4. Add 100 µl stop solution. Leave untouched for 5 minutes. Read at 450 nm.