11. Assay Procedure Continued…

8. Using a repeating dispenser, rapidly dispense 100µl of TMB Substrate **(Reagent 4)** into each well. Incubate the plate for **10 minutes**.

9. Add 100µl of Stop Solution **(Reagent 5)** to each well. To allow equal reaction times, the Stop Solution should be added to the wells in the same order as the TMB Substrate.

10. Read the optical density (OD) of each well at 450nm in a microplate reader within 10 minutes. A 620nm filter may be used as a reference wavelength.

12. Quality Control

Quality control data is supplied on the lot-specific QC certificate included in the kit.

Controls are intended to monitor for substantial reagent failure.

Any well positive by spectrophotometer but without visible colour should be cleaned on the underside and re-read. If OD-values below zero are observed, the wavelengths used should be verified, the reader re-blanked to air and the measurements repeated.

13. Interpretation of Results

Quantitative Results

Plot the optical density of each standard against its concentration and draw the best-fit curve through the points. Read the unknowns off this curve. Patients with active celiac disease have values of t-Tg IgA above 7 U/ml.

After several weeks on a gluten free diet, t-Tg antibody levels may become normal.

Normal Reference Range

IgA 0 - 7 U/ml

Ideally, each laboratory should establish its own normal and pathological range.

Qualitative Results

t-Tg IgA: Samples with ODs > than that of the 7U/ml standard are positive

14. Limitations of the Procedure

1. Due to the high incidence of IgA deficiency in celiac disease, some patients may show false negative results.

2. Results of this assay should be interpreted in conjunction with all available clinical information. Diagnosis should not rely solely on the t-Tg antibody results.

15. Performance Characteristics

56 samples from patients with known anti-endomysial antibody status, as determined by indirect immunofluorescence, were tested in the anti-t-Tg IgA ELISA. The performance characteristics of the assay, based on this study, are given in the table below.

Sensitivity	100%
Specificity	100%
Positive predictive value	100%
Negative predictive value	100%
Accuracy	100%

16. Reproducibility

Within Assay Precision

CV%: <5%

Between Assay Precision

CV%: <10%

17. Method Summary

- Dilute sera 1:100 with Sample Diluent **(Reagent 1)**
- Dispense Standards as required, the Positive and Negative Controls and the diluted sample into the microplate wells
- Incubate for **30 minutes** at room temperature.
- *Wash the wells three times*
- Dispense 100µl of Conjugate **(Reagent 3)** into each well
- Incubate at room temperature for **30 minutes**
- *Wash the wells four times*
- Add 100µl of TMB Substrate **(Reagent 4)** to each well
- Incubate at room temperature for **10 minutes**
- Add 100µl Stop Solution **(Reagent 5)** to each well
- Read the optical density at 450nm (single wavelength) or 450/620nm (dual wavelength).

18. Further Reading

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Sulkanen S *et al* (1998) Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. *Gastroenterology,* 115:1332-1328

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Marsh, M N (1997) Transglutaminase, gluten and coeliac disease: Food for thought. *Nature Medicine,* 3 (7), 725-726

Eden Research Park, Henry Crabb Road, Littleport, Cambridgeshire,
CB6 1SE, UK Tel+ 44(0)1353 862220 Fax+44(0)1353 863330
Email: support@elisa.co.uk Web: www.omegadiagnostics.com

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G·E·N·E·S·I·S Diagnostics

Recombinant Tissue Transgluatminase IgA ELISA Kit

Quantitative/qualitative assay for tissue transglutaminase IgA antibodies

Product Code: GD071

For *in vitro* Diagnostic Use

CE

1. Materials Included in the Kit

- **Microplate:** 12 X 8 break-apart strips (96 tests) pre-coated with recombinant human tissue transglutaminase, sealed in a foil bag with desiccant
- **Reagent 1**:**Sample Diluent** 150mM Tris-buffered saline, pH 7.2 with antimicrobial agent, (blue), 10ml, **concentrate** (x15)
- **Reagent 2: Wash Buffer** 100mM Tris-buffered saline with detergent, pH 7.2, 100ml, **concentrate** (x10)
- **Reagent 3: Conjugate** rabbit anti-human IgA (yellow) conjugated to horseradish peroxidase in protein stabilising solution and antimicrobial agent, 12ml, ready to use
- **Reagent 4: TMB Substrate** aqueous solution of TMB and hydrogen peroxide, 12ml, ready to use
- **Reagent 5: Stop Solution** 0.25M sulphuric acid, 12ml, ready to use
- **t-Tg IgA Standards:** 0, 7, 25, 50 & 100 U/ml, 1ml of 10mM Tris-buffered saline with human serum IgA antibodies to t-Tg, ready to use
- **Positive control:** 1ml of 10mM Tris-buffered saline containing human serum antibodies to t-Tg, ready to use.
- **Negative control:** 1ml of 10mM Tris-buffered saline containing normal human serum, ready to use.
- **Instructions for use**

2. Other Equipment Required

Test tubes for dilution • graduated cylinder for preparing wash buffer • precision pipettes and disposable tips to deliver 10µl, 100µl, 1ml • EIA microplate washer or multi-channel pipette or wash bottle • distilled or de-ionised water • absorbent paper • EIA microplate reader with 450nm and optional 620nm reference filter. Alternatively, a suitable, self-validated automated system may be used.

Instrumentation, whether manual or automated, should meet the following criteria: pipettes with better than 3% imprecision with no carry over between pipetting steps; microplate washers should remove 99% of fluid; automated machines should minimise time between washing and adding the next reagent.

3. Intended Use

The Tissue Transglutaminase IgA kit is a rapid ELISA method for the detection of circulating IgA antibodies to tissue transglutaminase (t-Tg). The kit has been designed to use the same protocol and sample diluent as used in the Genesis anti-gliadin IgG and IgA kits. The components of the kits are for *in vitro* diagnostic use only.

4. Explanation of the Test

Gluten-sensitive patients develop IgA and IgG antibodies to gliadin and to a component of the gut endomysium. Recently, tissue transglutaminase, a calcium-dependent enzyme that catalyzes the transamidation of specific polypeptide-bound glutamine residues, has been identified as the unknown endomysial antigen. Interestingly, gliadin is the preferred substrate for this enzyme creating antigenic neo-epitopes, which are thought to generate the immune response in genetically susceptible individuals.

The immunological detection of autoantibodies to t-Tg is a useful tool in the diagnosis and follow-up of celiac disease. The presence of autoantibodies to t-Tg closely correlates with the detection of anti-endomysial antibodies by indirect immunofluorescence. The ELISA method allows economical and rapid screening of large numbers of individuals for the presence of latent or sub clinical disease.

The assays use a recombinant human t-Tg preparation, which, in a number of studies, has been found to confer improved specificity over current guinea pig antigen based ELISAs.

5. Principle of the Test

Diluted serum samples are incubated with human recombinant t-Tg immobilised on microtitre wells. After washing away unbound serum components, rabbit anti-human IgA conjugated to horseradish peroxidase is added to the wells, and this binds to surface-bound antibodies in the second incubation. Unbound conjugate is removed by washing, and a solution containing 3,3',5,5'-tetramethylbenzidine (TMB) and enzyme substrate is added to trace specific antibody binding. Addition of Stop Solution terminates the reaction and provides the appropriate pH for colour development. The optical densities of the standards, controls and samples are measured using a microplate reader at 450nm.

6. Safety Precautions

1. All reagents in this kit are for *in vitro* diagnostic use only.
2. Only experienced laboratory personnel should use this test. The test protocol must be followed strictly.
3. CAUTION: the device contains material of human and animal origin and should be handled as a potential transmitter of diseases. All human source material used in the preparation of standards and control for this product have been tested and found negative by ELISA for antibodies to HIV, HbsAg and HCV. No test method, however, can offer complete assurance that infectious agents are absent. Therefore, all reagents containing human material should be handled as if potentially infectious. Operators should wear gloves and protective clothing when handling any patient sera or serum based products.
4. Reagents of this kit contain antimicrobial agents and the Substrate solution contains 3,3',5,5'-tetramethylbenzidine. Avoid contact with the skin and eyes. Rinse immediately with plenty of water if any contact occurs.
5. The Stop Solution contains 0.25M sulphuric acid. Avoid contact with skin and eyes. Rinse immediately with plenty of water if contact occurs.
6. Any liquid that has been brought into contact with potentially infectious material has to be discarded in a container with a disinfectant. Dispose of plates and specimens as clinical waste. Any unused reagents should be flushed away with copious amounts of water. Disposal must be performed in accordance with local legislation.

7. Technical Precautions

1. Strips and solutions should not be used if the foil bag is damaged or liquids have leaked.
2. Allow all reagents and the microplate to reach room temperature before use. Ensure that the microplate foil bag containing any unused strips is well sealed and contains the desiccant to avoid moisture. Store at 2 - 8°C after use.
3. The sample diluent X15 concentrate contains 0.09% sodium azide as preservative. Prepare sufficient working strength diluent for the assay run. However, if the working strength diluent is to be stored for more than 1 week, add sodium azide (0.9g/L). Store unused sample diluent concentrate and dilute sample diluent at 2 - 8°C.
4. When automating, consider excess volumes required for setting up the instrument and dead volume of robot pipette
5. Include the Positive and Negative Control in every test run to monitor for reagent stability and correct assay performance.
6. Strictly observe the indicated incubation times and temperature.
7. Ensure that no cross-contamination occurs between wells. Keep all pipettes and other equipment used for Conjugate completely separate from the TMB Substrate reagent.
8. When pipetting Conjugate or TMB Substrate, aliquots for the required numbers of wells should be taken to avoid multiple entry of pipette tips into the reagent bottles. Never pour unused reagents back into the original bottles.
9. Do not allow microwells to dry between incubation steps.
10. Strictly follow the described wash procedure. Insufficient washing may cause high background signal.
11. Avoid direct sunlight and exposure to heat sources during all incubation steps.
12. Replace colour-coded caps on their correct vials to avoid cross-contamination
13. It is important to dispense all samples and controls into the wells without delay. Therefore ensure that all samples are ready to dispense.

8. Shelf Life and Storage Conditions

On arrival, store the kit at 2 - 8°C. Once opened the kit is stable for 3 months (or until its expiry date if less than 3 months). Do not use kits beyond their expiry date. Do not freeze any kit component. The diluted Wash Buffer and Sample Diluent (see Technical Precautions) have a shelf life of 3 months if stored in a closed bottle at 2 - 8°C.

9. Specimen Collection and Storage

Serum and plasma samples may be used and should be stored at -20°C for long-term storage. Frozen samples must be mixed well after thawing and prior to testing. Repeated freezing and thawing can affect results. Addition of preservatives to the serum sample may adversely affect the results. Microbially contaminated, heat-treated or specimens containing particulate matter should not be used. Grossly haemolysed, icteric or lipaemic specimens should be avoided.

10. Preparation of Reagents

1. Dilute the Sample Diluent **(Reagent 1)** 1:14 in distilled water to make sufficient buffer for the assay run e.g. add 10 ml sample diluent concentrate to 140 ml water.

2. Dilute the Wash Buffer **(Reagent 2)** 1:9 in distilled water to make sufficient buffer for the assay run e.g. add 50ml wash buffer concentrate to 450ml water

11. Assay Procedure

1. Dilute patient samples 1:100 in diluted Sample Diluent (e.g. 10µl serum plus 1ml diluent).

2. Assemble the number of strips required for the assay.

3. For quantitative assays, dispense 100 µl of each Standard, the Negative and Positive Controls and the diluted patient samples into appropriate wells.

   For qualitative assays, dispense only the 7U/ml Standard.

4. Incubate for **30 minutes** at room temperature.

5. After 30 minutes, decant or aspirate the well contents and wash the wells 3 times using automated washing or the manual wash procedure (see below). Careful washing is the key to good results. **Do not allow the wells to dry out.**

   <u>Manual Wash Procedure</u>
   Empty the wells by inversion. Using a multi-channel pipette or wash bottle, fill the wells with Wash Buffer. Empty by inversion and blot the wells on absorbent paper. Repeat this wash process 2 more times.

6. Dispense 100µl of Conjugate **(Reagent 3)** into each well. Incubate the wells for **30 minutes** at room temperature.

7. After 30 minutes, discard the well contents and carefully wash the wells 4 times with Wash Buffer. Ensure that the wells are empty but do not allow to dry out.