Version 22-04/24

User's Manual

Anti-GAD ELISA Kit

REF **DEIA2289**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

Enzyme immunoassay for the determination of autoantibodies to Glutamic Acid Decarboxylase (GAD_{65}) in human serum.

General Description

Type 1 diabetes, also known as insulin-dependent diabetes mellitus (IDDM), results from a chronic autoimmune destruction of the insulinsecreting pancreatic beta cells, probably initiated by exposure of genetically susceptible host to environmental agents. Autoimmune destruction of beta cells is thought to be completely asymptomatic until 80-90% of the cells are lost. This process may take years to complete and may occur at any time in all ages.

During the preclinical phase, this autoimmune process is marked by circulating autoantibodies to beta cell antigens. These autoantibodies, such as anti-insulin (IAA), anti-glutamic acid decarboxylase (GAD) and antityrosine phosphatase ICA 512 (IA2), are present years before the onset of type 1 diabetes and prior to clinical symptoms.

GAD, the enzyme that catalyzes the conversion of glutamate to GABA, has been identified in two isoforms, molecular weight 65.000 (GAD_{65}) and 67.000 (GAD_{67}). Although GAD autoantibodies are found in type 1 diabetes and in the rare neurological disorder Stiff-man syndrome (SMS), the GAD autoantibodies profile in the two diseases differs.

Autoantibodies of SMS patients recognize a combination of linear and conformational epitopes of GAD while GAD_{65} autoantibodies in patients with type 1 diabetes are predominantly directed to the conformational epitopes. GAD_{65} autoantibodies (GAD_{65} Abs) are present in 70-80% of newly diagnosed patients with type 1 diabetes.

The combination of the autoantibodies to GAD_{65} and IA2 is highly relevant for risk assessment of type 1 diabetes in children and adolescence. These tests in combination are more sensitive and predictive than ICA in risk groups, e.g. relatives of patients with type 1 diabetes.

GAD_{65} Abs also occur in a subset of adults with type 2 diabetes. These patients can have pronounced hyperglycemia, and after therapy with oral hypoglycemic agents for several months to years they may become insulin dependent. Therefore, these patients are thought to have a slowly progressive form of type 1 diabetes, often called latent diabetes or latent autoimmune diabetes in adults (LADA).

The presence of GAD_{65} Abs in sera of such patients is a sensitive and specific marker for future insulin dependency.

Principles of Testing

The ELISA (Enzyme Linked Immunosorbent Assay) is an immunoassay for the determination of specific antibodies. The strips of the microtiter plate are coated with test-specific antigens. If antibodies are present in the patient's sample, they bind to the antigens. A second biotinylated antigen binds the immobilized antibody. The assay utilizes the ability of antibodies to act bivalently with immobilized and soluble antigens. Streptavidin conjugated with the enzyme peroxidase detects the generated immune complex. A colorless

substrate is converted into the colored product by the peroxidase. The signal intensity of the reaction product is proportional to the antibody activity in the sample. After stopping the signal intensity of the reaction product is measured photometrically.

Reagents And Materials Provided

1. **A. Microtiter plate:** 1 piece 12 breakable microtiter strips (ready-to-use), 8 wells per strip, each well coated with recombinant human GAD_{65}.
2. **Calibrator 0 – 5:** 6 × 1.0 mL. Colored dilutions of human serum (ready-to-use; contains ProClin 950) The antibody activities are indicated on the quality control certificate.
3. **CII. Positive control:** CONTROL +, 1 × 1.0 mL. Colored dilution of human serum (ready-to-use; contains ProClin 950) The antibody activity is indicated on the quality control certificate.
4. **C. Sample diluent:** 1 × 20 mL: Colored solution (ready-to-use; contains ProClin 950).
5. **H. GAD_{65}-Biotin:** 1 × 0.2 mL, Concentrated start reagent biotinylated GAD_{65} (contains sodium azide).
6. **J. Diluent for GAD_{65} - Biotin:** 1 × 20 mL, Solution (ready-to-use; contains ProClin 950).
7. **D. Streptavidin-peroxidase (SA-POD):** 1 × 0.2 mL, Concentrated streptavidin conjugated to horseradish peroxidase (100×).
8. **G. Diluent for SA-POD:** 1 × 20 mL, Solution (ready-to-use; contains ProClin 950).
9. **B. Wash buffer:** 1 × 100 mL, Concentrated solution (10×; contains ProClin 950).
10. **E. Substrate:** 1 × 15 mL, 3,3´,5,5´-Tetramethylbenzidine (ready-to-use).
11. **F. Stop solution:** 1 × 15 mL, 0.25 M Sulfuric acid (ready-to-use).
12. **Adhesive Foil:** 2 pieces.

Materials Required But Not Supplied

1. Common laboratory equipment
2. Precision pipettes (5 – 1000 µL), multi-channel pipettes (100 – 1000 µL) and disposable pipette tips
3. Graduated cylinders (100 – 1000 mL)
4. Vortex mixer or other rotators
5. Microtiter plate shaker
6. Microtiter plate washer or wash comb
7. Microtiter plate reader with optical filters for 450 nm and 620 nm or 690 nm
8. Adsorbent paper or paper towel
9. Distilled or de-ionized water

Storage

Upon receipt, all test components must be stored at 2 °C to 8 °C, preferably in the original kit box. If stored properly in their original containers, all components are stable until their expiry date

Specimen Collection And Preparation

1. **Sample Material:** The use of freshly collected serum from blood taken by venipuncture is recommended. The use of icteric, lipemic, hemolytic or bacterially contaminated samples should be avoided. Insoluble substances must be removed from the sample by centrifugation. Samples must not be thermally inactivated.
2. **Sample Storage:** Samples may be kept at 2 °C to 8 °C up to three days. Long-term storage requires -20 °C. Repeated freezing and thawing should be avoided. For multiple use, samples should be aliquoted and kept at -20 °C.

Reagent Preparation

All components including the microtiter plate must be brought to room temperature (RT: 18 °C to 25 °C) before use for at least 30 min. All liquid components must be mixed gently to ensure homogeneity.

1. **Microtiter Plate:** The microtiter plate is sealed in an aluminium bag. Unused test strips should always be stored refrigerated and protected from moisture with the desiccant in the properly sealed aluminum bag. Carefully resealed, the test strips can be used for 8 weeks after opening.
2. **Calibrators:** The calibrators are ready-to-use and must not be diluted any further. Calibrators must be used in each test run.
3. **Controls:** The positive control is ready to use and must not be diluted any further. Controls must be used in each test run. Laboratories can also validate their own control samples and use them alternatively.
4 **GAD_{65}-Biotin (H) and Diluent (J):** A sufficient amount of GAD_{65}-Biotin solution must be prepared by diluting x mL GAD_{65}-Biotin (H) with y mL diluent for GAD_{65}-Biotin (J) directly before use, For exact dilution volumes x and y see certificate of analysis supplied with the kit. The dilution ratio for manual and automated processing can be different. The GAD_{65}- Biotin solution prepared is to be used within one day and must not be stored.
5. **Streptavidin-peroxidase (D) and Diluent (G):** A sufficient amount of streptavidin-peroxidase solution must be prepared by diluting SA-POD concentrate (D) 1 + 99 (e. g. 0.1 mL SA-POD concentrate with 9.9 mL diluent for SA-POD (G). The SA-POD solution prepared is stable up to 4 weeks at 2 °C to 8 °C.
6. **Wash Buffer:** The wash buffer is concentrated and must be diluted 1:10 with distilled water before use (e. g. 100 mL + 900 mL). A sufficient amount of washing solution must be prepared. The diluted washing solution can be stored at 2 °C to 8 °C up to 30 days.
7. **Substrate:** The substrate is ready-to-use. Exposure of the substrate solution to strong light should be avoided.
8. **Stop Solution:** The stop solution is ready-to-use.

Assay Procedure

The indicated incubation times and temperatures must be adhered to and significant time shifts during pipetting samples and reagents must be avoided. The microtiter plate should be shortly shaken after addition of reagents.

1. Addition of dilution buffer: Add 100 µL ready-to-use dilution buffer into the wells of patient samples; leave the wells of calibrators and controls empty.
2. Addition of calibrators, controls and samples: Add 50 µL ready-to-use calibrators, controls and undiluted samples per well.
3. Incubation: Cover the plate and incubate for 60 min. at RT while shaking at 500 rpm on a plate shaker.

4. Preparation of reagents: Prepare sufficient volumes of reagents (B, D/G, H/J).
5. Wash cycle: Aspirate the solution and wash 3 times with 300 µL washing solution with at least 5 seconds soaking time each; dry by tapping the microtiter plate on a paper towel to remove any residual droplets.
6. Addition of start reagent: Add 100 µL of diluted GAD_{65}-Biotin solution (prepared from H and J) to each well.
7. Incubation: Cover the plate and incubate for 60 min. at RT while shaking at 500 rpm on a plate shaker.
8. Wash cycle: Aspirate the solution and wash 3 times with 300 µL washing solution with at least 5 seconds soaking time each; dry by tapping the microtiter plate on a paper towel to remove any residual droplets.
9. Addition of conjugate: Add 100 µL of diluted SA-POD (prepared from D and G) to each well.
10. Incubation: Cover the plate and incubate for 20 min. at RT while shaking at 500 rpm on a plate shaker.
11. Wash cycle: Aspirate the solution and wash 3 times with 300 µL washing solution with at least 5 seconds soaking time each; dry by tapping the microtiter plate on a paper towel to remove any residual droplets.
12. Addition of substrate: Add 100 µL ready-to-use substrate to each well and shake the plate shortly.
13. Incubation: Cover the plate and incubate for 20 min. in the dark at RT without shaking.
14. Addition of Stop Solution: Add 100 µL ready-to-use stop solution to each well and shake the plate shortly.
15. Analysis: Read optical density (OD) at 450 nm versus 620 or 690 nm within 30 min. after stopping the reaction.

Interpretation Of Results

A positive test result indicates the presence of specific antibodies. A negative result indicates the absence of specific antibodies, but does not exclude the possibility of an autoimmune reaction. In case of a borderline test result, a reliable evaluation is not possible.

Evaluation

1. **Metrological Traceability**

The immunoassay is calibrated using the international WHO reference preparation NIBSC code 97/550. Quantitative results are expressed in IU/mL.

2. **Quantitative Evaluation**

For generation of a standard curve, the optical signals (optical density, OD) of the calibrators are plotted against their antibody activities and correlated by a 4-parameter logistic (4 PL) fit. Antibody activities of unknown samples can be derived directly from their optical signals by use of the generated standard curve.

3 **Criteria of Validity**

Test runs are only valid if the following criteria of validity are fulfilled:

- OD CAL 0 < CAL 1 < CAL 2 < CAL 3 < CAL 4 < CAL 5

- OD CAL 5 > 1.2

- The positive control must be evaluated positive and present an antibody activity within the validity range indicated on the quality control certificate.

If these criteria are not met, the test is not valid and must be repeated.

4. **Troubleshooting**

In case of an invalid test run, the expiry dates and storage conditions, incubation times and temperatures, and precise calibration of all instruments used should be verified. If no reason for an invalid test run could be identified, please contact the supplier or manufacturer of the product.

5. **Reference Ranges**

The reference ranges are indicated below:

Antibody activity < 5 IU/mL negative

Antibody activity ≥ 5 IU/mL positive

As a result of different seroprevalences in individual regions, each laboratory should verify the reference ranges by own analysis and adapt, if necessary.

Detection Range

Reliable accuracy, trueness, precision, linearity and recovery of test results have been observed within the measurement range of the assay from the LoQ to the upper calibrator in comprehensive studies. Samples with test results above the upper calibrator should be reported as >max. Samples with test results below the LoQ should be reported as < min. If test results above the upper calibrator are observed, the samples may be tested at a higher dilution. The resulting antibody activity must be multiplied with the additional dilution factor.

Detection Limit

Limit of Blank (LoB) < 0.5 IU/mL

Limit of Quantitation (LoQ) < 1.0 IU/mL

Sensitivity

Sensitivity: 90.5 %

Specificity: 94.7 %

Precautions

The instructions for use must be carefully read before use. They are valid only for the present product with the given composition and must be strictly followed to ensure reliable test results.

Deviations can lead to erroneous test results. Components must not be exchanged by test reagents of different lots or of other manufacturers.

Contamination of reagents must be avoided by use of aseptic techniques when removing aliquots from the vials.

After use, reagent vials must be tightly closed with their corresponding caps. Cross-contamination of samples or reagents can lead to inconsistent test results and must be avoided by use of consistent pipetting techniques.

Exposure of reagents to strong light must be avoided throughout the entire test procedure and storage.

Insufficient washing will result in poor precision and elevated measurement signals. After each washing step any residual fluid has to be removed completely.

Limitations

In the rare neurological disorder, Stiff-man Syndrome (SMS) round 60 % of patients have GAD_{65} Abs in their serum. GAD_{65} Abs from patients with SMS have much higher titers compared with those of patients with type 1 diabetes. For this reason sera from patients with suspicion of SMS should be prediluted 1:50 or 1:100 with GAD_{65} Abs negative sera. GAD_{65} Abs occur also in cerebrospinal fluid of patients with SMS.

The interpretation of test results must always be considered in combination with the clinical picture of the patient. The diagnosis should not be based on the results of a sole diagnostic method. All clinical and laboratory findings should be evaluated to state a diagnosis. For confirmation, further investigations should be carried out.