Anti GAD

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Enzyme immunoassay for the quantitative determination of GAD in human serum

1. INTRODUCTION

Type 1 diabetes, also known as insulin-dependent diabetes mellitus (IDDM), results from a chronic autoimmune destruction of the insulin-secreting 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 anti-tyrosine 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 (GAD65) and 67.000 (GAD67). 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 GAD65 autoantibodies in patients with type 1 diabetes are predominantly directed to the conformational epitopes. GAD65 autoantibodies (GAD65 Abs) are present in 70-80% of newly diagnosed patients with type 1 diabetes. The combination of the autoantibodies to GAD65 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. GAD65 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 GAD65 Abs in sera of such patients is a sensitive and specific marker for future insulin dependency.

2. INTENDED USE

Anti-GAD kit is for research only qualitative ELISA test for the quantitative determination of autoantibodies to glutamic acid decarboxylase(GAD65 Abs) in human serum of prediabetic high risk individuals as well as IDDM diabetic patients.Anti GAD kit is intended for laboratory use only.

3. PRINCIPLE OF THE ASSAY

The assay system uses the ability of GAD65 Abs acting divalently and forming a bridge between immobilized GAD65 and liquid-phase GAD65-Biotin. In the first step GAD65 Ab from the sample bind to GAD65 coated on the microtiter plate. In a second stepGAD65-Biotin binds to this complex. The bound GAD65-Biotin correlates with the amount of GAD65 Abs in patient's serum.Unbound GAD65-Biotin is removed by washing.The bound GAD65-Biotin could be quantified by addition of Streptavidin-peroxidase and a chromogenic substrate (TMB) and readingthe optical density (OD) at 450 nm.

4. MATERIALS

4.1. Reagents supplied

Anti GAD Coated Wells: 12 breakapart 8-well snap-off strips coated with anti-GAD; in resealable aluminium foil.
Stop Solution: 1 bottle containing 12 ml sulphuric acid, 0.25 mol/l (avoid any skin contact), ready to use
20x conc. Streptavidin-peroxidase: 1 bottle containing 0.7 ml 20x conc. Streptavidin-peroxidase
Streptavidin-peroxidase diluent: 1 bottle containing 15ml, ready to use
GAD65 Biotin: 3 bottles, lyophilised
Biotin diluent: 2 bottles containing 15 ml each, ready to use
TMB Substrate Solution: 1 bottle containing 15 ml 3, 3´, 5, 5´-tetramethylbenzidine (H_2O_2-TMB 0.26 g/l) (avoid any skin contact), ready to use
Wash solution: 1 bottle containing 125 ml (10x conc.)
anti GAD Standards: 5 bottles, 0.7 ml each, ready to use
Standard 1: 5 IU/ml
Standard 2: 18 IU/ml
Standard 3: 35 IU/ml
Standard 4: 120 IU/ml

Standard 5: 250 IU/ml
Negative Control: 1 bottle containing 0.7 ml, ready to use
Positive Control: 1 bottle containing 0.7 ml, ready to use

4.2. Materials supplied

1 Strip holder
1 Cover foils
1 Test protocol
1 Distribution and identification plan

4.3. Materials and Equipment needed

ELISA microwell plate reader, equipped for the measurement of absorbance at 450 nm
Manual or automatic equipment for rinsing wells
Pipettes to deliver volumes between 10 and 1000 µl
Vortex tube mixer
Distilled water
Disposable tubes
Timer

5. STABILITY AND STORAGE

The reagents are stable up to the expiry date stated on the label when stored at 2...8 °C in the dark.

6. REAGENT PREPARATION

It is very important to bring all reagents, samples and standards to room temperature (22…28°C) before starting the test run!

6.1. Coated snap-off Strips

The ready to use break apart snap-off strips are coated with anti-GAD antibodies. Store at 2…8 °C. Open the bag only when it is at room temperature. *Immediately after removal of strips, the remaining strips should be resealed in the aluminium foil along with the desiccant supplied and stored at 2…8 °C; stability until expiry date. Do not remove the adhesive sheets on the unused strips.*

6.2. Biotin

Prepare a sufficient amount of GAD65-Biotin solution by reconstitution of one vial lyophilized GAD65-Biotin with 5.5 mL diluent for GAD65-Biotin directly prior to use. The GAD65-Biotin solution can be store at 2-8°C for 3 days.

6.3. Streptavidin-peroxidase

Prepare a sufficient amount of Streptavidin-peroxidase solution by diluting the Streptavidin-peroxidase 20X Concentrate 1 + 19 with Streptavidin-peroxidase diluent (i.e 0.25 mL of Streptavidin-peroxidase concentrate with 4.75 mL of diluent). The solution prepared is stable up to 16 weeks at 2-8°C.

6.4. anti-GAD Standards/controls

The standards/controls are ready to use and have the following concentration of anti-GAD:

Standard 1: 5 IU/ml
Standard 2: 18 IU/ml
Standard 3: 35 IU/ml
Standard 4: 120 IU/ml
Standard 5: 250 IU/ml
Negative control
Positive control

6.5. TMB Substrate Solution

The bottle contains 15 ml of a tetramethylbenzidine/hydrogen peroxide system. The reagent is ready to use and has to be stored at 2...8°C in the dark. *The solution should be colourless or could have a slight blue tinge. If the substrate turns into blue, it may have become contaminated and should be thrown away*.

6.6. Stop Solution

The bottle contains 15 ml 0.15 M sulphuric acid solution (R 36/38, S 26). This ready to use solution has to be stored at 2...8°C.

6.7. Wash Solution

Prepare a sufficient amount of washing solution by diluting the 10X Concentrated Wash Solution 1 + 9 with distilled or deionized water. For

example, dilute 50 mL of the Concentrate Wash with 450 mL of distilled water. The solution should be free of crystals before dilution, otherwise dissolve by warming up to max 37°C. The diluted washing solution can be stored at 2-8°C up to 30 days.

7. SPECIMEN COLLECTION AND PREPARATION

The determination of anti-GAD can be performed in serum.Store the sample at -20°C if the determination is not performed on the same day as the sample collection.

8. ASSAY PROCEDURE

8.1. Test Preparation

Please read the test protocol carefully **before** performing the assay. Result reliability depends on strict adherence to the test protocol as described. Prior to commencing the assay, the distribution and identification plan for all specimens and controls should be carefully established on the result sheet supplied in the kit. Select the required number of microtiter strips or wells and insert them into the holder. Please allocate at least:

1 well (e.g. A1) for the substrate blank 2 wells (e.g. B1+C1) control negative 2 wells (e.g. D1+E1) for standard 1 2 wells (e.g. F1+G1) for standard 2 2 wells (e.g. H1+A2) for standard 3 2 wells (e.g. B2+C2) for standard 4 2 wells (e.g. D2+E2) for standard 5 2 wells (e.g. F2+G2) control positive

Reagent	Standard	Sample or Control	Blank
Sample		25 µL	
Standard S1-S5	25 µl		
Controls		25 µL	
Cover the plate with a plastic film and incubate at room temperature (22-28°C) for 1 hour while shaking > 500 rpm. Remove the content from each well and wash the wells 3 times with 300 L of diluted Wash Solution.			
GAD65 Biotin	100 µL	100 µL	
Cover the plate with a plastic film and incubate at room temperature (22-28°C) for 1 hour while shaking > 500 rpm. Remove the contents from each well and wash the wells 3 times with 300 L of diluted Wash Solution.			
Streptavidin peroxidase	100 µL	100 µL	
Cover the plate with a plastic film and incubate at room temperature (22-28°C) for 20 minutes while shaking > 500 rpm. Remove the contents from each well and wash the wells 3 times with 300 L of diluted Wash Solution.			
TMB Substrate Solution	100 µL	100 µL	100 µL
Incubate at room temperature (22-28°C) for 20 minutes in the dark.			
Stop Solution	100 µL	100 µL	100 µl
Shake the microplate gently. Read the absorbance (E) at 450 nm against Blank within 5 min after adding the Stop Solution.			

9.1. Calculation of results

The standard curve is established by plotting the mean OD-values of the calibrators 1 -5 on the ordinate, y-axis, versus theirrespective GAD65 Ab-concentrations on the abscissa, x-axis. In addition negative control (CI) should be used (see below).The GAD65 Abs concentrations of the controls and the unknown samples are directly read off in IU/ml from the measured OD450values.The anti-GAD kit may be used also with Computer Assisted Analysis using software able to curves with spline smoothing fit.Example:

Sample	OD (a) 450 nm	OD (b) 450 nm	OD (mean)	IU/mL
Control CI	0.145	0.121	0.133	1
Standard 1	0.244	0.283	0.264	5
Standard 2	0.351	0.391	0.371	18
Standard 3	0.684	0.740	0.712	35
Standard 4	1.765	1.868	1.817	120

Standard 5	3.397	3.702	3.550	250
Control CII				
Patient 1	0.850	0.857	0.854	41.8

Reference Values

anti-GAD	
negative	< 5.0 IU/mL
positive	≥ 5.0 IU/mL

It is recommended that each laboratory establishes its own normal and pathological reference ranges for serum anti-GAD65 antibodies levels as usually done for other diagnostic parameters, too. Therefore, the abovementioned reference values provide only a guide.

10. SPECIFIC PERFORMANCE CHARACTERISTICS

10.1. Precision

Intra Assay Variation Within run variation was determined by replicate 12 times four different sera with values in the range of standard curve. The within assay variability is ≤7.6%

Inter Assay Variation Between run variation was determined by replicate the measurements of one control serum with different lots of kits and/or different mix of lots of reagents. The between assay variability is ≤8.2%.

10.2. Calibration

The anti-GAD kit is calibrated against the WHO reference preparation NIBSC 97/550 and concentrations of GAD65 Abs are therefore expressed in IU/mL.

10.3. Linearity

On the basis of the heterogeneous nature of the autoantibody population and in view of epitope specificity and affinity of the autoantibodies the theoretical values expected by dilution with GAD65 Abs free human serum do not correspond with the measured concentrations in some cases.

10.4.Detection Limits

The analytical sensitivity of Anti GAD kit was established to be 0.24 IU/mL.

10.5. Analytic Sensitivity / Specificity

Using a cut-off of 5 IU/mL, Anti GAD kit shows a sensitivity of 88.6% and specificity of 92.3%, regarding patients with newly onset type 1 diabetes.
11.6. Analytic Specificity

11. PRECAUTIONS AND WARNINGS

Please adhere strictly to the sequence of pipetting steps provided in this protocol.
All reagents should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated.
Allow all kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
Do not interchange kit components from different lots. The expiry dates printed on the labels of the box and of the vials must be observed. Do not use any kit component beyond their expiry date.
Avoid the exp WARNING: the conjugate reagent is designed to ensure maximum dose sensitivity and may be contaminated by external agents if not used properly; therefore, it is recommended to use disposable consumables (tips, bottles, trays, etc.). For divided doses, take the exact amount of conjugate needed and do not re-introduce any waste product into the original bottle. In addition, for doses dispensed with the aid of automatic and semi-automatic devices, before using the conjugate, it is advisable to clean the fluid handling system, ensuring that the procedures of washing, deproteinization and decontamination are effective in avoiding contamination of the conjugate; this procedure is highly recommended when the kit is processed using analyzers which are not equipped with disposable tips.
If you use automated equipment is your responsibility to make sure that the kit has been appropriately tested.
The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background.
It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate
Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore,

the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.

Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera.

Maximum precision is required for reconstitution and dispensation of the reagents.

Samples microbiologically contaminated should not be used in the assay. Highly lipemeic or haemolysed specimens should similarly not be used

Plate readers measure vertically. Do not touch the bottom of the wells.

This kit is intended for research use by professional persons only.

Use appropriate personal protective equipment while working with the reagents provided.

All human source material used in the preparation of standards and controls for this product has been tested and found negative for antibody to HIV 1&2, HbsAg, and HCV. No test method however can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, the Standard and the Controls should be handled in the same manner as potentially infectious material.

Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy and the bovine protein has been obtained from countries not infected by BSE, but these materials should be handled as potentially infectious.

Some reagents contain small amounts of Sodium Azide (NaN3) or Proclin 300R as preservatives. Avoid the contact with skin or mucosa.

Sodium Azide may be toxic if ingested or absorbed through the skin or eyes; moreover it may react with lead or copper plumbing to form potentially explosive metal azides. If you use a sink to remove the reagents, allow scroll through large amounts of water to prevent azide build-up.

The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.

The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.

12.1. Disposal Considerations

Residues of chemicals and preparations are generally considered as hazardous waste. The disposal of this kind of waste is regulated through national and regional laws and regulations. Contact your local authorities or waste management companies which will give advice on how to dispose hazardous waste.

13. LITERATURE

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SCHEME OF THE ASSAY

Anti-GAD

Test Preparation

Prepare reagents and samples as described.Establish the distribution and identification plan for all specimens and controls on the resultsheet supplied in the kit.

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