IBL INTERNATIONAL

Instructions for Use

Anti-GAD ELISA

Enzymimmunoassay for the quantitative determination of autoantibodies to Glutamic Acid Decarboxylase (GAD65 Ab) in human serum.

REF **MP53051**

Σ **96**

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

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INTENDED USE

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 (IA_2), 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 IA_2 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.

LITERATURE

- Batstra M, HJ Anstoot & P Herbrink: Prediction and diagnosis of type 1 diabetes using ß-cell autoantibodies; Clin Lab 2001, 47:497-507
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- Pozzilli P, S Manfrini & L Monetini: Biochemical markers of type 1 diabetes; clinival use, Scand J Clin Lab Invest 2001;61:38-44
- Schernthaner G, S Hink, HP Kopp, B Muzyka, G Streit & A Krois: Progress in the characterization of slowly progressive autoimmune diabetes in adult patients (LADA or type1,5 diabetes); Exp Clin Endocrinol diabetes 2001 Suppl 2: S94-S108
- Winter WE, N Harris & D Schatz: Immunological markers in the diagnosis and prediction of autoimmune Type 1a diabetes; Clinical Diabetes 2002, 20: 183-191

PRINCIPLE of the TEST

Anti-GAD is an enzyme immunoassay for the quantitative determination of autoantibodies to glutamic acid decarboxylase (GAD_{65} Abs) in human serum.
The assay system uses the ability of GAD_{65} Abs acting divalently and forming a bridge between immobilized GAD_{65} and liquid-phase GAD_{65}-Biotin. In the first step GAD_{65} Ab from the sample bind to GAD_{65} coated on the microtiter plate. In a second step GAD_{65}-Biotin binds to this complex. The bound GAD_{65}-Biotin correlates with the amount of GAD_{65} Abs in patient's serum. Unbound GAD_{65}-Biotin is removed by washing.
The bound GAD_{65}-Biotin could be quantified by addition of Streptavidin- peroxidase and a colorogenic substrate (TMB) and reading the optical density (OD) at 450 nm.

PATIENT SAMPLES

Specimen collection and storage

Blood is taken by venipuncture. After clotting, the serum is separated by centrifugation. Do not use lipaemic or grossly hemolytic serum samples. Plasma should not be used.

The samples may be kept at 2 - 8 °C up to three days. Long-term storage requires - 20 °C.
Repeated freezing and thawing should be avoided. For multiple use, initially aliquot samples and keep at - 20 °C.

TEST CONPONENTS for 96 DETERMINATIONS

A MP	**Microtiter plate** 12 breakable strips per 8 wells coated with human recombinant GAD_{65}		vacuum sealed with desiccant
B WASHB	**Concentrated wash buffer** sufficient for 1000 mL		**100 mL** concentrate
D CONJ	**Streptavidin-peroxidase** (SA-POD) sufficient for 14.0 mL		**0.7 mL** concentrate
E SUB	**Substrate** (3,3´,5,5´-Tetramethylbenzidin)		**15 mL** ready for use
F STOP	**Stop solution** (0.25 M sulfuric acid)		**12 mL** ready for use
G BUF D	**Diluent for SA-POD (D)**		**15 mL** ready for use
H START	**GAD_{65}-Biotin**		**3 vials** lyophilized
J BUF H	**Diluent for GAD_{65}-Biotin (H)**		**2 x 15 mL** ready for use colored red
C I CONTROL	**negative control**		**0.25 mL** ready for use
C II CONTROL	**positive control** concentration: see leaflet		**0.25 mL** ready for use
1 - 5 CAL	**calibrators** concentrations see leaflet		**5 vials** 0.25 mL each, ready for use

Materials required

- Precision pipettes 10 - 100 µL
- Multi-channel pipette
- Disposable pipette tips
- 8 channel wash comb or microplate washer
- Micro plate reader with optical filters for 450 nm and 620 or 690 nm
- Graduated cylinders
- Distilled or de-ionized water
- Absorbent paper or paper towel
- foil

Size and storage

Anti-GAD has been designed for 96 determinations. This is sufficient for the analysis of 40 unknown samples as well as for calibrators and control sera assayed in duplicates.

The expiry date of each component is reported on its respective label, that of the complete kit on the box label.

Upon receipt, all components of Anti-GAD have to be kept at 2 - 8 °C, preferably in the original kit box.

Preparation before use

Allow samples to reach room temperature prior to assay. Take care to agitate serum samples gently in order to ensure homogeneity.

Please, handle carefully with the following components:

A Allow the sealed microplate to reach room temperature before opening. Unused wells should be stored refrigerated and protected from moisture in the original bag carefully resealed for 16 weeks.

B Prepare a sufficient amount of washing solution by diluting the concentrated wash buffer (B) 1 + 9 with distilled or de-ionized water. For example, dilute 50 mL of the concentrate with 450 mL of distilled water. B 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.

D Prepare a sufficient amount of Streptavidin-peroxidase solution by diluting SA-POD concentrate (D) 1 + 19 (0.25 mL SA-POD concentrate with 4.75 mL diluent for SA-POD (G)**.** The SA-POD solution prepared is stable up to 16 weeks at 2 - 8 °C.

E Avoid exposure of substrate solution (E) to light.

H Prepare a sufficient amount of GAD_{65}-Biotin solution by reconstitution of one vial lyophilized GAD_{65}-Biotin (H) with 5.5 mL diluent for GAD_{65}-Biotin (J) directly prior to use. The GAD_{65}-Biotin solution can be store at 2 - 8 °C for 3 days.

ASSAYS PROCEDURE

- Duplicates are recommended.

1. Pipette into the corresponding wells according to assay scheme
   - **25 µL** negative control (C I) and calibrators (1 - 5)
   - **25 µL** patient's samples and control serum (C II).
2. Cover the plate and incubate for **60 min** at room temperature (18 - 25 °C) while shaking > 200 rpm.
3. Aspirate or "flick out" by striking the wells sharply onto absorbent paper to remove any residual droplets. Wash **3 times** with **300 µL** washing solution (diluted from B) with 5 seconds soaking time each.
4. Add **100 µL** of reconstituted GAD_{65}-Biotin solution (prepared from H and J) to each well.
5. Cover the plate and incubate for **60 min** at room temperature (18 - 25 °C) while shaking > 200 rpm.
6. Aspirate or "flick out" by striking the wells sharply onto absorbent paper to remove any residual droplets. Wash **3 times** with **300 µL** washing solution (diluted from B) with 5 seconds soaking time each.
7. Add **100 µL** reconstituted SA-POD (prepared from D and G) to each well.
8. Cover the plate and incubate for **20 min** at room temperature (18 - 25 °C) while shaking > 200 rpm.
9. Aspirate or "flick out" by striking the wells sharply onto absorbent paper to remove any residual droplets. Wash **3 times** with **300 µL** washing solution (diluted from B) with 5 seconds soaking time each.
10. Add **100 µL** substrate solution (**E**) to each well and shake shortly.
11. Incubate for **20 min** in the **dark** at room temperature.
12. Add **100 µL** stop solution (**F**) after exact **20 min** to each well. Shake the plates for 5 seconds > 200 rpm.
13. Read the optical density **at 450 nm** versus **620 or 690 nm within 5 min** after adding the stop solution.

Please note that the washing procedure is crucial. Insufficient washing will result to poor precision and falsely elevated OD readings. Without shaking the ODs will be measured about 20 % lower with a loss of sensitivity.

DATA PROCESSING

The standard curve is established by plotting the mean OD-values of the calibrators 1 - 5 on the ordinate, y-axis, versus their respective GAD_{65} b-concentrations on the abscissa, x-axis. In addition negative control (CI) should be used (see below).

The GAD_{65} Abs concentrations of the controls and the unknown samples are directly read off in IU/mL from the measured OD_{450} values.

Very high concentrations of GAD_{65} Abs could be measured by reading absorbencies at 405 nm instead of 450 nm.

Anti-GAD may be used also with Computer Assisted Analysis using software able to plot lin/log curves with spline smoothing or sigmoid fit.

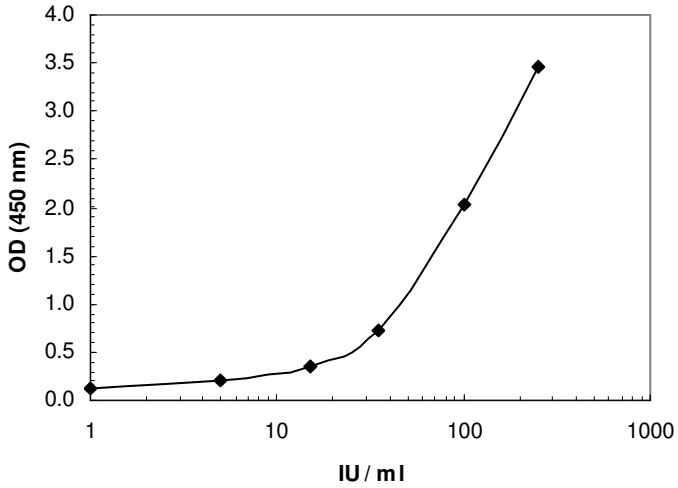
TYPICAL EXAMPLE

Do not use for evaluation!

Sample	OD (a) 450 nm	OD (b) 450 nm	OD (mean)	IU / mL
Control **CI**	0.145	0.121	0.133	**1**
Calibrator **1**	0.244	0.283	0.264	**5**
Calibrator **2**	0.351	0.391	0.371	**15**
Calibrator **3**	0.684	0.740	0.712	**35**
Calibrator **4**	1.765	1.868	1.817	**100**
Standard **5**	3.397	3.702	3.550	**250**
Control **CII**	---	---	---	---
Patient 1	0.850	0.857	0.854	41.8

STANDARD CURVE

Typical example



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-GAD_{65} antibodies levels as usually done for other diagnostic parameters, too. Therefore, the abovementioned reference values provide only a guide.

CHARACTERISTIC ASSAY DATA

Calibration

The Anti-GAD is calibrated against the WHO reference preparation NIBSC 97/550 and concentrations of GAD_{65} Abs are there-fore expressed in IU/mL.

Linearity

On the basic 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 GAD_{65} Abs free human serum do not correspond with the measured concentrations in some cases.

Specificity and sensitivity

Using a cut-off of 5 IU/mL the Anti-GAD shows a sensitivity of 92.3 % and specificity of 98.6 %, regarding patients with newly onset type 1 diabetes.

Detection limits

The analytical sensitivity (lower detection limit, 0 ± 3 SD) was stablished to be 0.8 IU/mL.
The functional sensitivity was measured as 20 % of inter-assay CV at 4 IU/mL.

Intra- and inter-assay variation

Intra-assay			Inter-assay		
Sample no.	Mean Concentration (IU/mL)	CV (%)	Sample no.	Mean Concentration (IU/mL)	CV (%)
1	16	4	5	5	14
2	60	4	6	42	7
3	151	4	7	99	3
4	256	3	8	237	25

LIMITATIONS of the METHOD

Healthy individuals should be tested negative by using the Anti-GAD. However, GAD_{65} Abs may also be present in apparently healthy persons.

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. That's the reason why sera from patients with suspicion of SMS should be prediluted 1:50 or 1:100 with GAD_{65} Abs negative sera. In patients with SMS GAD_{65} Abs occur also in cerebrospinal fluid.

Any clinical diagnosis should not be based on the results of in vitro diagnostic method alone. Physicians are supposed to consider all clinical and laboratory findings possible to state a diagnosis.

Anti-GAD

ASSAY SCHEME

Bring all reagents to room temperature. Gently mix all reagents to ensure homogeneity.

Step	Activity	Material	CI / CAL	C II	Patients 1, 2 etc.
1	Pipette	Samples	25 µL	25 µL	25 µL
2	Incubate	Plate	**1 hour** at room temperature **with shaking (> 200 rpm)**		
3	Aspirate or decant		put sharply onto absorbent tissue		
	Pipette	Washing solution	3 x 300 µL	3 x 300 µL	3 x 300 µL
4	Pipette	GAD_{65}-Biotin solution	100 µL	100 µL	100 µL
5	Incubate	Plate	**1 hour** at room temperature **with shaking (> 200 rpm)**		
6	Aspirate or decant		put sharply onto absorbent tissue		
	Pipette	Washing solution	3 x 300 µL	3 x 300 µL	3 x 300 µL
7	Pipette	SA-POD solution	100 µL	100 µL	100 µL
8	Incubate	Plate	**20 min** at room temperature **with shaking (> 200 rpm)**		
9	Aspirate or decant		put sharply onto absorbent tissue		
	Pipette	Washing solution	3 x 300 µL	3 x 300 µL	3 x 300 µL
10	Pipette	Substrate	100 µL	100 µL	100 µL
11	Incubate	Plate	**20 min** at room temperature **in the dark**		
12	Pipette and mix	Stop solution	100 µL	100 µL	100 µL
13	Measure OD		at 450 nm versus 620 nm (or 690 nm) within 5 min		

SAFETY PRECAUTIONS

- **This kit is for in vitro use only**. Follow the working instructions carefully.
- The expiration dates stated on the respective labels are to be observed. The same relates to the stability stated for reconstituted reagents.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept at 2 - 8 °C before use in the original shipping container.
- Some of the reagents contain small amounts (< 0.1 % w/w) sodium azide as preservatives. They must not be swallowed or allowed to come into contact with skin or mucosa.
- Source materials derived from human body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed
  - Do not smoke, eat or drink while handling kit material
  - Always use protective gloves
  - Never pipette material by mouth
  - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.
- In any case GLP should be applied with all general and individual regulations to the use of this kit.

Symbols / Symbole / Symbôles / Símbolos / Símbolos / Σύμβολα

REF	Cat.-No.: / Kat.-Nr.: / No.- Cat.: / Cat.-No.: / N.º Cat.: / N.–Cat.: / Αριθμός-Κατ.:
LOT	Lot-No.: / Chargen-Bez.: / No. Lot: / Lot-No.: / Lote N.º: / Lotto n.: / Αριθμός -Παραγωγή:
	Use by: / Verwendbar bis: / Utiliser à: / Usado por: / Usar até: / Da utilizzare entro: / Χρησιμοποιείται από:
	No. of Tests: / Kitgröße: / Nb. de Tests: / No. de Determ.: / N.º de Testes: / Quantità dei tests: / Αριθμός εξετάσεων:
CONC	Concentrate / Konzentrat / Concentré / Concentrar / Concentrado / Concentrato / Συμπύκνωμα
LYO	Lyophilized / Lyophilisat / Lyophilisé / Liofilizado / Liofilizado / Liofilizzato / Λυοφιλιασμένο
IVD	In Vitro Diagnostic Medical Device. / In-vitro-Diagnostikum. / Appareil Médical pour Diagnostics In Vitro. / Dispositivo Médico para Diagnóstico In Vitro. / Equipamento Médico de Diagnóstico In Vitro. / Dispositivo Medico Diagnostico In vitro. / Ιατρική συσκευή για In-Vitro Διάγνωση.
	Evaluation kit. / Nur für Leistungsbewertungszwecke. / Kit pour évaluation. / Juego de Reactivos para Evaluació. / Kit de avaliação. / Kit di evaluazione. / Κιτ Αξιολόγησης.
	Read instructions before use. / Arbeitsanleitung lesen. / Lire la fiche technique avant emploi. / Lea las instrucciones antes de usar. / Ler as instruções antes de usar. / Leggere le istruzioni prima dell'uso. / Διαβάστε τις οδηγίες πριν την χρήση.
	Keep away from heat or direct sun light. / Vor Hitze und direkter Sonneneinstrahlung schützen. / Garder à l'abri de la chaleur et de toute exposition lumineuse. / Manténgase alejado del calor o la luz solar directa. / Manter longe do calor ou luz solar directa. / Non esporre ai raggi solari. / Να φυλάσσεται μακριά από θερμότητα και άμεση επαφή με το φως του ηλίου.
	Store at: / Lagern bei: / Stocker à: / Almacene a: / Armazenar a: / Conservare a: / Αποθήκευση στους:
	Manufacturer: / Hersteller: / Fabricant: / Productor: / Fabricante: / Fabbricante: / Παραγωγός:
	Caution! / Vorsicht! / Attention! / ¡Precaución! / Cuidado! / Attenzione! / Προσοχή!

Symbols of the kit components see MATERIALS SUPPLIED.
Die Symbole der Komponenten sind im Kapitel KOMPONENTEN DES KITS beschrieben.
Voir MATERIEL FOURNI pour les symbôles des composants du kit.
Símbolos de los componentes del juego de reactivos, vea MATERIALES SUMINISTRADOS.
Para símbolos dos componentes do kit ver MATERIAIS FORNECIDOS.
Per i simboli dei componenti del kit si veda COMPONENTI DEL KIT.
Για τα σύμβολα των συστατικών του κιτ συμβουλευτείτε το ΠΑΡΕΧΟΜΕΝΑ ΥΛΙΚΑ.

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