CE

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DCM082-6
Ed. 12/2023

Anti GAD

for routine analysis

Quantitative ELISA test for the detection of circulating autoantibodies against GAD antigens.

RUO

LOT
See external label

2°C 8°C

Σ = 96 tests

REF DKO082

INTENDED USE

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

1. CLINICAL SIGNIFICANCE

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. PRINCIPLE

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 step GAD65-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 then reading the optical density (OD) at 450 nm.

Anti GAD antibodies concentration in the sample is calculated through a calibration curve.

3. REAGENTS, MATERIALS AND INSTRUMENTATION

3.1. Reagents and materials supplied in the kit

1. Calibrators (5 vials, 0.7 mL each)

CAL1	REF DCE002/8207-0
CAL2	REF DCE002/8208-0
CAL3	REF DCE002/8209-0
CAL4	REF DCE002/8210-0
CAL5	REF DCE002/8211-0

2. Controls (2 vials, 0.7 mL each, ready to use)

Negative control	REF DCE045/8201-0
Positive control	REF DCE045/8202-0

3. 20X Conc. Streptavidin-peroxidase (1 vial, 0.7 mL) REF DCE041/8241-0

4. Streptavidin-peroxidase diluent (1 vial, 15 mL) REF DCE048/8248-0

5. GAD65 Biotin (3 vials, lyophilised) REF DCE019/8219-0

6. Biotin diluent (2 vials, 15 mL each) REF DCE047/8247-0

7. Coated Microplate (1 breakable microplate)
Purified GAD adsorbed on the microplate
REF DCE002/8203-0

8. TMB Substrate (1 vial, 15 mL)
H_2O_2-TMB (0,26 g/L) *(avoid any skin contact)*
REF DCE004/8204-0

9. Stop Solution (1 vial, 12 mL)
Sulphuric acid 0.25M *(avoid any skin contact)*
REF DCE005/8205-0

10. 10X Conc. Wash Solution (1 vial, 125 mL)
REF DCE006/8206-0

3.2. Reagents necessary not supplied
Distilled or deionized water.

3.3. Auxiliary materials and instrumentation
Automatic dispenser.
Microplates reader (450 nm, 620-630 nm)

Notes
Store all reagents between 2÷8°C in the dark.
Open the bag of reagent 7 (Coated Microplate) only when it is at room temperature and close it immediately after use; once opened, it is stable until expiry date of the kit.

4. WARNINGS
- This kit is intended for research use by professional persons only. Not for internal or external use in Humans or Animals.
- Use appropriate personal protective equipment while working with the reagents provided.
- Follow Good Laboratory Practice (GLP) for handling blood products.
- All human source material used in the preparation of the reagents 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, Calibrators and 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 (NaN_3) or ProClin™ 300 as preservative. Avoid contact with skin or mucosa.
- Classification according to Regulation (EC) No. 1272/2008 [CLP]

20X Conc. Streptavidin-peroxidase
Skin sensitivity, Category 1

Warning

Hazard statements:
H317 - May cause an allergic skin reaction.

Precautionary statements:
P261 - Avoid breathing dust / fume / gas / mist / vapours / spray.
P272 - Contaminated work clothing should not be allowed out of the workplace
P280 - Wear protective gloves/ protective clothing / eye protection / face protection / hearing protection.
P302+P352 - IF ON SKIN: Wash with plenty of soap and water
P333+P313 - If skin irritation or rash occurs: Get medical advice/attention.
P362+P364 - Take off contaminated clothing and wash it before reuse.
P501 - Dispose of contents/container to hazardous or special waste collection point, in accordance with local, regional, national and/or international regulation.

TMB Substrate
Reproductive toxicity, Category 1B

Danger

Hazard statements:
H360D - May damage the unborn child.

Precautionary statements:
P202 - Do not handle until all safety precautions have been read and understood.
P280 - Wear protective gloves/ protective clothing / eye protection / face protection / hearing protection.
P308+P313 - IF exposed or concerned: Get medical advice/attention.
P501 - Dispose of contents/container to hazardous or special waste collection point, in accordance with local, regional, national and/or international regulation.

Streptavidin-peroxidase diluent
Hazard statements:
EUH208 - May produce an allergic skin reaction.

Precautionary statements:
N/A

- 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.

- Avoid the exposure of reagent TMB/H_2O_2 to directed sunlight, metals or oxidants. Do not freeze the solution.

5. PRECAUTIONS

- Please adhere strictly to the sequence of pipetting steps provided in this protocol. The performance data represented here were obtained using specific reagents listed in this Instruction For Use.
- All reagents should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as 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 date printed on box and vials labels must be observed. Do not use any kit component beyond their expiry date.
- **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.
  For this purpose, Dia.Metra supplies a separate decontamination reagent for cleaning needles.
- If you use automated equipment, the user has the 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, highly lipemeic or haemolysed should not be used in the assay.
- Plate readers measure vertically. Do not touch the bottom of the wells.

6. PROCEDURE

6.1. Preparation of the 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.

6.2. Preparation of the Wash Solution

Prepare a sufficient amount of washing solution by diluting the 10X Conc. Wash Solution 1:10 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.

6.3. Preparation of the Streptavidin-peroxidase

Prepare a sufficient amount of Streptavidin-peroxidase solution by diluting the Streptavidin-peroxidase 20X Concentrate 1:20 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. Procedure

- **Allow all reagents to reach room temperature (22-28°C) for at least 30 minutes.** At the end of the assay store immediately the reagents at 2-8°C: avoid long exposure to room temperature.
- Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C.
- To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.
- As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the calibration curve (C_1-C_5), two for each Control, two for each sample, one for Blank.

Reagent	Calibrator	Sample/ Controls	Blank
Sample		**25 µL**	
Calibrator C_1-C_5	**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 µL of diluted Wash Solution. **Important note**: during each washing step, gently shake the plate for 5 seconds and remove excess solution by tapping the inverted plate on an absorbent paper towel.			
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 µL of diluted Wash Solution. **Washing**: follow the same indications of the previous point.			
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 µL of diluted Wash Solution. **Washing**: follow the same indications of the previous point.			
TMB Substrate	**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 a reference wavelength of 620-630 nm or against Blank within 5 minutes.			

7. RESULTS

7.1. Calibration curve

The calibration curve is established by plotting the mean OD-values of the calibrators 1 - 5 on the ordinate, y-axis, versus their respective 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 OD450 values.

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
Calibrator 1	0.244	0.283	0.264	5
Calibrator 2	0.351	0.391	0.371	18
Calibrator 3	0.684	0.740	0.712	35
Calibrator 4	1.765	1.868	1.817	120
Calibrator 5	3.397	3.702	3.550	250
Control CII	---	---	---	---
Patient 1	0.850	0.857	0.854	41.8

7.2. Reference Values

Anti GAD	
Negative	< 5.0 IU/mL
Positive	≥ 5.0 IU/mL

Please pay attention to the fact that the determination of a range of expected values for a “normal” population in a given method is dependent on many factors, such as specificity and sensitivity of the method used and type of population under investigation. Therefore each laboratory should consider the range given by the Manufacurer as a general indication and produce their own range of expected values based on the indigenous population where the laboratory works.

8. CHARACTERISTICS

8.1. 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.

8.2. 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.

8.3. Specificity and Sensitivity

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.

8.4. Detection Limits

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

8.5. Intra and inter-assay variations

8.5.1. Intra-Assay

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

8.5.2. Inter-Assay

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%.

9. WASTE MANAGEMENT

Reagents must be disposed off in accordance with local regulations.

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DiaMetra	Packaging Information Sheet	Mod. PIS000-1

IVD	DE ES FR GB IT PT	In vitro Diagnostikum Producto sanitario para diagnóstico In vitro Dispositif medical de diagnostic in vitro In vitro Diagnostic Medical Device Dispositivo medico-diagnostico in vitro Dispositivos medicos de diagnostico in vitro		DE ES FR GB IT PT	Hergestellt von Elaborado por Fabriqué par Manufacturer Produttore Produzido por
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	DE ES FR GB IT PT	Vor direkter sonneneinstrahlung schützen Mantener alejado de la luz solar Tenir à l'écart de la lumière du soleil Keep away from sunlight Tenere lontano dalla luce solare Mantenha longe da luz solar			

SUGGERIMENTI PER LA RISOLUZIONE DEI PROBLEMI/TROUBLESHOOTING

ERRORE CAUSE POSSIBILI/ SUGGERIMENTI

Nessuna reazione colorimetrica del saggio

- mancata dispensazione del coniugato
- contaminazione del coniugato e/o del Substrato
- errori nell'esecuzione del saggio (es. Dispensazione accidentale dei reagenti in sequenza errata o provenienti da flaconi sbagliati, etc.)

Reazione troppo blanda (OD troppo basse)

- coniugato non idoneo (es. non proveniente dal kit originale)
- tempo di incubazione troppo breve, temperatura di incubazione troppa bassa

Reazione troppo intensa (OD troppo alte)

- coniugato non idoneo (es. non proveniente dal kit originale)
- tempo di incubazione troppo lungo, temperatura di incubazione troppa alta
- qualità scadente dell'acqua usata per la soluzione di lavaggio (basso grado di deionizzazione,)
- lavaggi insufficienti (coniugato non completamente rimosso)

Valori inspiegabilmente fuori scala

- contaminazione di pipette, puntali o contenitori- lavaggi insufficienti (coniugato non completamente rimosso)

CV% intrasaggio elevato

- reagenti e/o strip non portate a temperatura ambiente prima dell'uso
- il lavatore per micropiastre non lava correttamente (suggerimento: pulire la testa del lavatore)

CV% intersaggio elevato

- condizioni di incubazione non costanti (tempo o temperatura)
- controlli e campioni non dispensati allo stesso tempo (con gli stessi intervalli) (controllare la sequenza di dispensazione)
- variabilità intrinseca degli operatori

ERROR POSSIBLE CAUSES / SUGGESTIONS

No colorimetric reaction

- no conjugate pipetted reaction after addition
- contamination of conjugates and/or of substrate
- errors in performing the assay procedure (e.g. accidental pipetting of reagents in a wrong sequence or from the wrong vial, etc.)

Too low reaction (too low ODs)

- incorrect conjugate (e.g. not from original kit)
- incubation time too short, incubation temperature too low

Too high reaction (too high ODs)

- incorrect conjugate (e.g. not from original kit)
- incubation time too long, incubation temperature too high
- water quality for wash buffer insufficient (low grade of deionization)
- insufficient washing (conjugates not properly removed)

Unexplainable outliers

- contamination of pipettes, tips or containers

insufficient washing (conjugates not properly removed) too high within-run

- reagents and/or strips not pre-warmed to CV% Room Temperature prior to use
- plate washer is not washing correctly (suggestion: clean washer head)

too high between-run - incubation conditions not constant (time, CV % temperature)

- controls and samples not dispensed at the same time (with the same intervals) (check pipetting order)
- person-related variation

ERROR / POSIBLES CAUSAS / SUGERENCIAS

No se produce ninguna reacción colorimétrica del ensayo

- no se ha dispensado el conjugado
- contaminación del conjugado y/o del substrato
- errores en la ejecución del ensayo (p. ej., dispensación accidental de los reactivos en orden incorrecto o procedentes de frascos equivocados, etc.)

Reacción escasa (DO demasiado bajas)

- conjugado no idóneo (p. ej., no procedente del kit original)
- tiempo de incubación demasiado corto, temperatura de incubación demasiado baja

Reacción demasiado intensa (DO demasiado altas)

- conjugado no idóneo (p. ej., no procedente del kit original)
- tiempo de incubación demasiado largo, temperatura de incubación demasiado alta
- calidad escasa del agua usada para la solución de lavado (bajo grado de desionización)
- lavados insuficientes (el conjugado no se ha retirado completamente)

Valores inexplicablemente fuera de escala

- contaminación de pipetas, puntas o contenedores- lavados insuficientes (el conjugado no se ha retirado completamente)

CV% intraensayo elevado

- los reactivos y/o tiras no se encontraban a temperatura ambiente antes del uso
- el lavador de microplacas no funciona correctamente (sugerencia: limpiar el cabezal del lavador)

CV% interensayo elevado

- condiciones de incubación no constantes (tiempo o temperatura)
- controles y muestras no dispensados al mismo tiempo (con los mismos intervalos) (controlar la secuencia de dispensación)
- variación en función de los operadores

ERREUR CAUSES POSSIBLES / SUGGESTIONS

Aucune réaction colorimétrique de l'essai

- non distribution du conjugué
- contamination du conjugué et/ou du substrat
- erreurs dans l'exécution du dosage (par ex., distribution accidentelle des réactifs dans le mauvais ordre ou en provenance des mauvais flacons, etc.)

Réaction trop faible (DO trop basse)

- conjugué non approprié (par ex., ne provenant pas du coffret original)
- temps d'incubation trop court, température d'incubation trop basse

Réaction trop intense (DO trop élevée)

- conjugué non approprié (par ex., ne provenant pas du coffret original)
- temps d'incubation trop long, température d'incubation trop élevée
- mauvaise qualité de l'eau utilisée pour la solution de lavage (bas degré de déionisation)
- lavages insuffisants (conjugué non entièrement éliminé)

Valeurs inexplicablement hors plage

- contamination des pipettes, embouts ou récipients - lavages insuffisants (conjugué non entièrement éliminé)

CV% intra-essai élevé

- les réactifs et/ou les bandes n'ont pas atteint la température ambiante avant usage
- le laveur de microplaques ne lave pas correctement (suggestion : nettoyer la tête du laveur)

CV% inter-essai élevé

- conditions d'incubation non constantes (temps ou température)
- contrôles et échantillons non distribués en même temps (avec les mêmes intervalles) (contrôler l'ordre de distribution)
- variabilité intrinsèque des opérateurs