DIAGNOSTIKA GMBH

Instructions for Use

GAD_{65} Antibody ELISA

Enzyme Immuno Assay for the Quantitative Determination of Antibodies against Glutamic Acid Decarboxylase in Serum

CE

IVD

REF EA104/96

12 x 8

2 – 8 °C

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Contents

Symbols

I V D	In-Vitro-Diagnostic Device	CE	EC Declaration of Conformity
CONT	Contents		Expiry Date
LOT	Lot Number	+2 +8 °C	Store
	Manufactured by	Σ	Sufficient for
REF	Catalogue Number		Consult Instructions

1. Introduction and Principle of the Test

Insulin-dependent diabetes mellitus (IDDM) is characterised by the presence of several distinct circulating autoantibodies including autoantibodies to insulin and autoantibodies to glutamic acid decarboxylase (GAD). Two isoforms of GAD of molecular weights 65,000 (GAD 65) and 67,000 (GAD 67) have been identified. GAD 65 is the predominant form found in human islets and has been shown to be a major target for autoantibodies in IDDM.

The GAD_{65} Antibody ELISA depends on the ability of GAD autoantibodies to act divalently and form a bridge between GAD coated on ELISA plate wells and liquid phase GAD-biotin. The GAD-biotin bound is is then quantitated by addition of streptavidin peroxidase and a colorogenic substrate (TMB) with reading of final absorbance at 2 wavelengths (450 and 405 nm) to obtain maximum measuring range (0-2,000 units per ml of WHO reference preparation NIBSC 97/550).

Note:
European patent 1448 993 B1, Chinese patent ZL02822274.1, Indian patent 226484, Japanese patent 5711449 and US patent 8,129,132 B1 apply. Also, US patents 6,682,906 B1 and 6,277,586 B1 (licenced to RSR) apply.

2. Precautions

- For in vitro use only.
- Some reagents contain sodium azide as preservative. Avoid skin contact.
- All reagents of human origin used in this kit are tested for HIV I/II antibodies, HCV and HBsAg and found to be negative. However, because no test method can offer complete assurance that infectious agents are absent, these reagents should be handled as potentially biohazardous materials.
- Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy but these materials should be handled as potentially infectious.

3. Sample Collection and Storage

Serum should be used in the assay. Sera to be analysed should be assayed soon after separation or stored (preferably in aliquots) at 2 - 8 °C for one week or at -20 °C for longer periods.

About 70 µl is sufficient for one assay. Subsequent freezing and thawing or increase in storage temperature must be avoided. Incorrect storage of serum samples can lead to loss of anti-GAD_{65} autoantibodies. Do not use grossly haemolysed or lipaemic serum samples. Do not use plasma in the assay.

When required, thaw test sera at room temperature and mix gently to ensure homogeneity. Centrifuge the serum prior to assay (preferably for 5 min at 10-15,000 g in a microfuge) to remove any particulate matter. Please do not omit this centrifugation step if sera are cloudy or contain particulates.

On arrival, store the kit at 2-8 °C. Once opened the kit is stable until its expiry date. For stability of prepared reagents refer to Preparation of Reagents.

4. Contents of the Kit

4.1 **MT strips** **STRIPS** 12 strips
8 wells per strip
coated with human recombinant GAD_{65},
after opening the pouch, place in the self seal plastic bag provided, store at 2-8°C and use within 16 weeks

4.2 **Calibrator 1 – 6** **CAL 1 – 6** 6 vials
0.7 ml each, ready for use

Concentrations (NIBSC 97/550 units):

Calibrator	1	2	3	4	5	6
U/ml	5	18	35	120	250	2,000

4.3 **Positive Control** **CON +** 1 vial
0.7 ml, ready for use
values for the control are given on the vial label

4.4 **Negative Control** **CON -** 1 vial
0.7 ml, ready for use

4.5 **GAD_{65} Biotin** **GAD-BIOTIN** 3 vials
freeze-dried; reconstitute with 5.5 ml buffer (coloured red)
prior to use, store at 2-8°C and use within 3 days of reconstitution

4.6 **Reconstitution Buffer** **RECONST** 2 vial
15 ml, coloured red, ready for use
for reconstituting GAD_{65} biotin

4.7 **Streptavidin-peroxidase** **SA-POD** 1 vial
0.7 ml; 20 x concentrated;
dilute 20 x with SA-POD Diluent prior to use;
store at 2-8°C and use within 16 weeks

4.8 **SA-POD Diluent** **DIL** 1 vial
15 ml, ready for use
for reconstituting SA-POD

4.9 **Substrate** **SUB** 1 vial
15 ml tetramethyl benzidine (TMB), ready for use

4.10 **Wash Buffer** **WASH** 1 vial
125 ml, 10 x concentrated
dilute to 1 litre with pure water before use.
Store at 2-8 °C after dilution

4.11 **Stop Solution** **STOPP** 1 vial
12 ml, ready for use
0.5 M sulphuric acid

Additional materials and equipment required but not provided:

- Pipettes for 25 µl, 50 µl, 100 µl
- Orbital shaker (up to 500 rpm)
- Pure water
- Microtiter plate reader (405 and 450 nm)

5. Test Procedure

5.1. Preparation of Reagents

MT strips
Before opening a packet of strip wells, allow it to stand at room temperature for at least 30 minutes. After opening, keep any unused wells in the original foil packet (reseal with adhesive tape) and in the self-seal plastic bag with the desiccant provided. Store at 2-8 °C and use within 16 weeks.

GAD_{65}-Biotin
Reconstitute the contents of one vial with 5.5 ml Reconstitution Buffer (coloured red) prior to use. Store at 2-8°C for up to 3 days after reconstitution.

Streptavidin-Peroxidase (SA-POD)
Dilute 20 x with Streptavidin-Peroxidase (SA-POD) Diluent prior to use. Store at 2-8°C for up to 16 weeks after reconstitution.

Wash Buffer
Dilute the contents of one bottle to 1 litre with pure water before use. Store at 2-8°C after dilution until expiry date of the kit.

5.2. Assay Procedure

Calculate the number of individual ELISA plate wells needed for the assay. Allow all the reagents supplied, including the appropriate number of packets of strips to reach room temperature (at least 30 min), remove the number of strip wells required and fit them firmly into the frame provided. Negative and Positive Controls in duplicate must always be included in each assay run.

1. Pipette each 25 µl of Calibrator 1 - 6, Negative and Positive Controls and test sera into the appropriate wells (duplicates are recommended).
2. Cover the frame and incubate for 1 hours at room temperature on an ELISA plate shaker (shaking at 500 rpm).

3. During this 1 hour incubation, reconstitute the GAD_{65} Biotin, dilute the required amount of Streptavidin-Peroxidase (SA-POD) and dilute the concentrated Wash Buffer.

4. After the 1-hour incubation, aspirate or discard the samples from the wells, add 300 µl of Wash Buffer and aspirate or discard again. Repeat two more times. Tap the inverted wells gently on a clean dry absorbent surface to remove any droplets of Wash Buffer.

5. Pipette 100 µl of reconstituted GAD_{65} biotin into each well, cover the frame and incubate for 1 hour at room temperature on an ELISA plate shaker (shaking at 500 rpm).

6. After the 1-hour incubation with GAD_{65} biotin, aspirate or discard the reagent from the wells, add 300 µl of Wash Buffer and aspirate or discard again. Repeat two more times. Tap the inverted wells gently on a clean dry absorbent surface to remove any droplets of Wash Buffer.

7. Pipette 100 µl of SA-POD into each well, cover the frame and incubate for 20 minutes at room temperature on an ELISA plate shaker (shaking at 500 rpm).

8. Aspirate or discard the reagent from the wells and wash three times with Wash Buffer followed by one wash with pure water to remove any foam from the wells. If a plate washing machine is used, wash 3 times with Wash Buffer only (i.e. omit water wash). Tap the inverted wells gently on a clean dry absorbent surface to remove any droplets.

9. Pipette 100 µl of Substrate (TMB) into each well and incubate for 20 minutes at room temperature in the dark <u>without</u> shaking during which time a blue colour will develop.

10. Stop the substrate reaction by addition of 100 µl of Stop Solution to each well (this will cause the blue colour to turn yellow), cover the frame and shake the plate for about 5 seconds on a plate shaker to ensure uniformity of the solution in each well.

11. Within 15 minutes read the absorbance at 450 nm and 405 nm using an ELISA plate reader blanked against a well containing 100 µl Substrate plus 100 µl of Stop Solution.

6. Calculation of Results

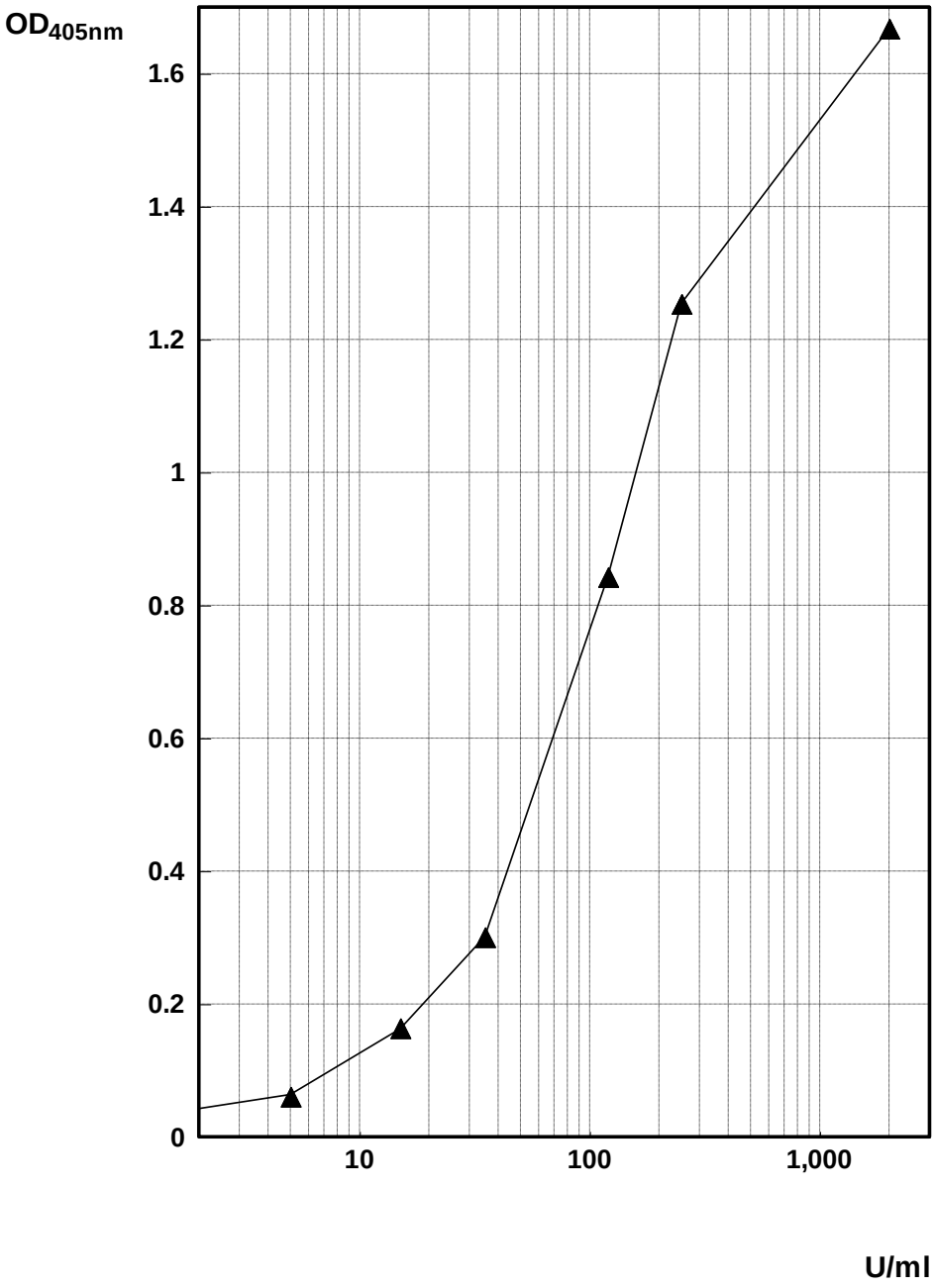
A standard curve can be plotted with absorbance at 450 nm or 405 nm at the vertical axis and $\log_{10}$ Standards concentration on the horizontal axis. Other data reduction procedures can also be used.

Typical Example

In the presence of the negative control, absorbance values at 450 nm should be about 0.1 increasing to above 4 with the with the higher concentration calibrators. Reading at 405 nm allows quantitation of the high absorbances as shown in the table. Low values should be read off the 450 nm curve.

Typical results are shown in the following table.

Calibrator U/ml	$OD_{450\ nm}$	$OD_{405\ nm}$
Negative Control	0.035	0.012
5	0.199	0.061
18	0.527	0.164
35	0.975	0.301
120	2.794	0.843
250	4.264	1.254
2,000	5.671	1.668



7. Test Characteristics

Assay Cut Off

Negative	< 5 U/ml
Positive	≥ 5 U/ml

Cinical Evaluation

Clinical Specificity and Sensitivity
In the DASP 2005 study the GADAb ELISA kit achieved 98% (n=100) specificity and 92% (n=50) sensitivity.

Lower Detection Limit
The kit negative control was assayed 20 times and the mean and standard deviation calculated. The lower detection limit at +2 standard deviations was 0.57 U/ml.

Clinical Accuracy
Analysis of sera from patients with autoimmune diseases other than type 1 DM disease indicated no interference from autoantibodies to thyroglobulin or thyroid peroxidase (n=10) or TSH receptor (n=20). One sample positive for dsDNA (n=10) and one sample positive for rheumatoid factor (n=30) were positive for GAD Ab.

Inter-Assay Precision

Sample	U/ml (n=20)	CV (%)
A	97	5.7
B	21	5.2
C	5.7	6.4

Intra-Assay Precision

Sample	U/ml (n=25)	CV (%)
1	97	7.3
2	20	8.5
3	7.0	3.5

Interference
No interference was observed when samples were spiked with the following materials; haemoglobin at up to 5 mg/ml, bilirubin at up to 20 mg/dl or Intralipid up to 3000 mg/dl.

8. Literature

H. Brooking et al
A Sensitive non-isotopic assay for GAD_{65} autoantibodies
Clinica Chimica Acta 2003 331:55-59

S. Chen et al
Sensitive non-isotopic assays for autoantibodies to IA2 and to a combination of both IA2 and GAD_{65}.
Clinica Chimica Acta 2005 357:74-83

E. Nilson et al
Calcium addition to EDTA plasma eliminates falsely positive results in the RSR GADAb ELISA.
Clinica Chimica Acta 388 (2008) 130-134

K. Rahmati et al
A Comparison of Serum and EDTA Plasma in the Measurement of Glutamic Acid Decarboxylase Autoantibodies (GADA) and Autoantibodies to Islet Antigen-2 (IA-2A) Using the RSR Radioimmunoassay (RIA) and Enzyme Linked Immunosorbent Assay (ELISA) Kits.
Clin. Lab. 2008 54:227-235

C. Törn et al
Diabetes Antibody Standardization Program: evaluation of assays for autoantibodies to glutamic acid decarboxylase and islet antigen-2.
Diabetologia 2008 51:846-852

Pipetting Scheme
GAD_{65} Antibody ELISA

		B_0	Calibrator	Positive Control	Patients
Calibrator 1 - 6	µl		25		
Positive Control	µl			25	
Negative Control	µl	25			
Patient Sample	µl				25

Cover and incubate for 1 hours at RT on a shaker (500 rpm)

Aspirate / discard and wash three times with each 300 µl Wash Buffer

GAD_{65}-Biotin	µl	100	100	100	100

Cover and incubate for 1 hour at RT on a shaker (500 rpm)

Aspirate / discard and wash three times with each 300 µl Wash Buffer

SA-POD	µl	100	100	100	100

Cover and incubate for 20 minutes at RT on a shaker (500 rpm)

Aspirate / discard and wash three times with each 300 µl Wash Buffer
Wash once with 300 µl pure water

TMB-Substrate	µl	100	100	100	100

Cover and incubate for 20 minutes at RT in the dark without shaking

Stop Solution	µl	100	100	100	100

5 sec shaking on an ELISA plate shaker

Reading of absorbance at 450 nm and 405 nm
within 15 minutes after stopping