BioVendor
Research
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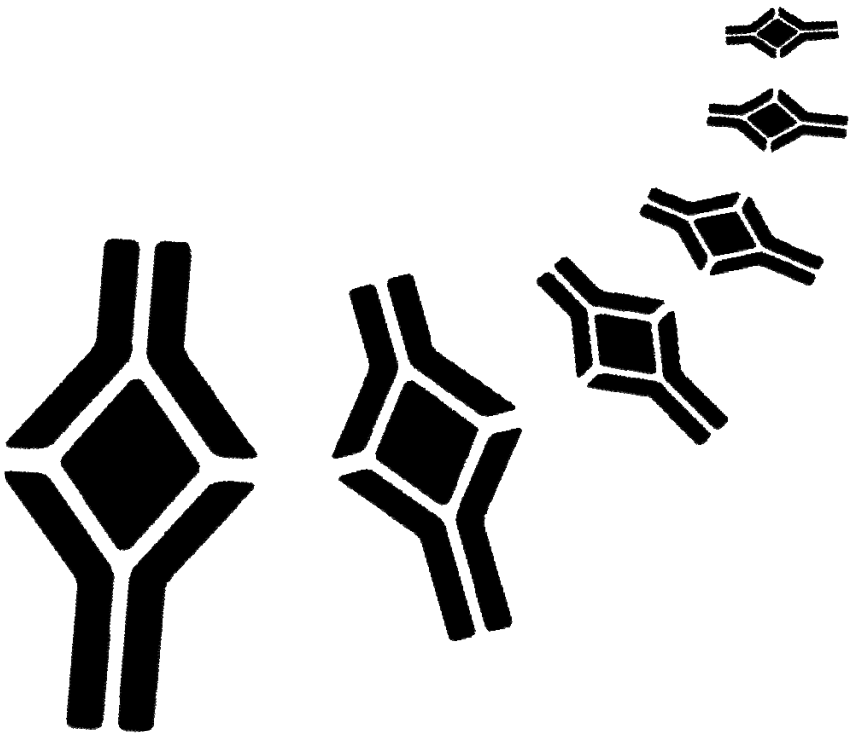


GAD_{65} Autoantibody ELISA

Product Data Sheet

Cat. No.: RGDE/96R

For Research Use Only

CONTENTS

» This kit is manufactured by: BioVendor – Laboratorní medicína, a.s.

» Use only the current version of Product Data Sheet enclosed with the kit!

1 INTENDED USE

The GAD_{65} autoantibody (GAD Ab) ELISA kit is intended for use by professional persons only, for the quantitative determination of GAD autoantibodies in human serum.
Autoantibodies to pancreatic beta cell antigens are important serological markers of type 1 diabetes mellitus (type 1 DM). The antigens recognised by these antibodies include insulin, glutamic acid decarboxylase (GAD_{65} kDa isoform) and an islet cell antigen IA-2 or ICA-512.

2 ASSAY PRINCIPLE

In GAD Ab ELISA, GAD autoantibodies in patients' sera, calibrators and controls are allowed to interact with GAD_{65} coated onto ELISA plate wells. After a 1 hour incubation, the samples are discarded leaving GAD autoantibodies bound to the immobilised GAD_{65} on the plate. GAD Biotin is added in a 2nd incubation step where, through the ability of GAD autoantibodies in the samples to act divalently, a bridge is formed between GAD immobilised on the plate and GAD Biotin. The amount of GAD Biotin bound is then determined in a 3rd incubation step by addition of Streptavidin Peroxidase, which binds specifically to Biotin. Excess, unbound Streptavidin Peroxidase is then washed away and addition of 3,3',5,5' – tetramethylbenzidine (TMB) results in formation of a blue colour. This reaction is stopped by addition of stop solution causing the well contents to turn yellow. The absorbance of the yellow reaction mixture at 450nm and 405nm is then read using an ELISA plate reader.
A higher absorbance indicates the presence of GAD autoantibody in the test sample. Reading at 405nm allows quantitation of high absorbances. Low values (less than 10 units per mL) should be read off the 450 nm calibration curve.

3 STORAGE AND PREPARATION OF SERUM SAMPLES

Sera to be analysed should be assayed soon after separation or stored, preferably in aliquots, at or below –20°C. 50 µL is sufficient for one assay (duplicate 25 µL determinations). Repeated freeze thawing or increases in storage temperature should be avoided. Do not use lipaemic or haemolysed serum samples. Do not use plasma in the assay. When required, thaw test sera at room temperature and mix gently to ensure homogeneity. Centrifuge serum prior to assay (preferably for 5 min at 10-15,000 g in a microfuge) to remove particulate matter. Please do not omit this centrifugation step if sera are cloudy or contain particulates.

4 MATERIALS REQUIRED AND NOT SUPPLIED

Pipettes capable of dispensing 25 µL and 100 µL.
Means of measuring out various volumes to reconstitute or dilute reagents.
Pure water.
ELISA plate reader suitable for 96 well formats and capable of measuring at 450nm and 405nm.
ELISA Plate shaker, capable of 500 shakes/min (not an orbital shaker).

5 PREPARATION OF REAGENTS SUPPLIED

A GAD_{65} Coated Wells
12 breakapart strips of 8 wells (96 in total) in a frame and sealed in foil bag.

Fit stripwells firmly into frame provided
After opening return any unused wells to the original foil packet and seal. Then place foil bag in the self-seal plastic bag with desiccant provided, and store at 2-8°C for up to 16 weeks.

B1-6 Calibrators
5, 18, 35, 120, 250, 2000 U/mL
(units are NIBSC 97/550)
6 x 0.7 mL
Ready for use

C Positive control
(see certificate of analysis for concentration range)
0.7 mL
Ready for use

D Negative control
0.7 mL
Ready for use

E GAD_{65} Biotin
3 vials
Lyophilised

Reconstitute each vial with 5.5 mL GAD Biotin reconstitution buffer (F). When more than one vial is used, pool the vials and mix gently before use. Store at 2 – 8°C for up to 3 days after reconstitution.

F Buffer for GAD-Biotin
2 x 15 mL, coloured red
Ready for use

G Streptavidin Peroxidase (SA-POD)
1 x 0.7 mL
Concentrated

Dilute 1 in 20 with diluent for diluting SA-POD (H). For example, 0.5 mL (G) + 9.5 mL (H). Store at 2 – 8°C for up to 16 weeks after dilution.

H Diluent for SA-POD
15 mL
Ready for use

I Peroxidase Substrate (TMB)
15 mL
Ready for use

J Concentrated wash solution
125 mL
Concentrated

Dilute 10 X with pure water before use. Store at 2 – 8°C up to kit expiry date.

K Stop solution
12 mL
Ready for use

6 ASSAY PROCEDURE

Allow all reagents to stand at room temperature for at least 30 minutes before use. A repeating Eppendorf type pipette is recommended for steps 4, 7, 10 and 11.

1. Pipette 25 µL of patient sera, calibrators (B1-6) and controls (C and D) into respective wells, in duplicate.
2. Cover the frame and shake the wells for 1 hour at room temperature on an ELISA plate shaker (500 shakes per min.).
3. Use an ELISA plate washer to aspirate and wash the wells three times with diluted wash solution (J). If a plate washer is not available, discard the well contents by briskly inverting the frame of stripwells over a suitable receptacle, wash three times manually and finally tap the inverted wells gently on a clean dry absorbent surface.
4. Pipette 100 µL of reconstituted GAD Biotin (E) into each well. Avoid splashing the material out of the wells during addition.
5. Cover the plate, and incubate at room temperature for 1 hour on an ELISA plate shaker (500 shakes per min).
6. Repeat wash step 3.
7. Pipette 100 µL of diluted Streptavidin Peroxidase (G) into each well.
8. Cover the plate and incubate at room temperature for 20 minutes on an ELISA plate shaker (500 shakes per min).
9. Repeat wash step 3. If manual washing is being carried out use one additional wash step with pure water (to remove any foam) before finally tapping the inverted wells dry,
10. Pipette 100 µL of TMB (I) into each well and incubate in the dark at room temperature for 20 minutes without shaking.
11. Pipette 100 µL stop solution (K) to each well and shake the plate for approximately 5 seconds on a plate shaker. Ensure substrate incubations are the same for each well.
12. Read the absorbance of each well at 450nm and 405nm using an ELISA plate reader, blanked against a well containing 100 µL of TMB (I) and 100 µL Stop solution (K) only.

7 RESULTS ANALYSIS

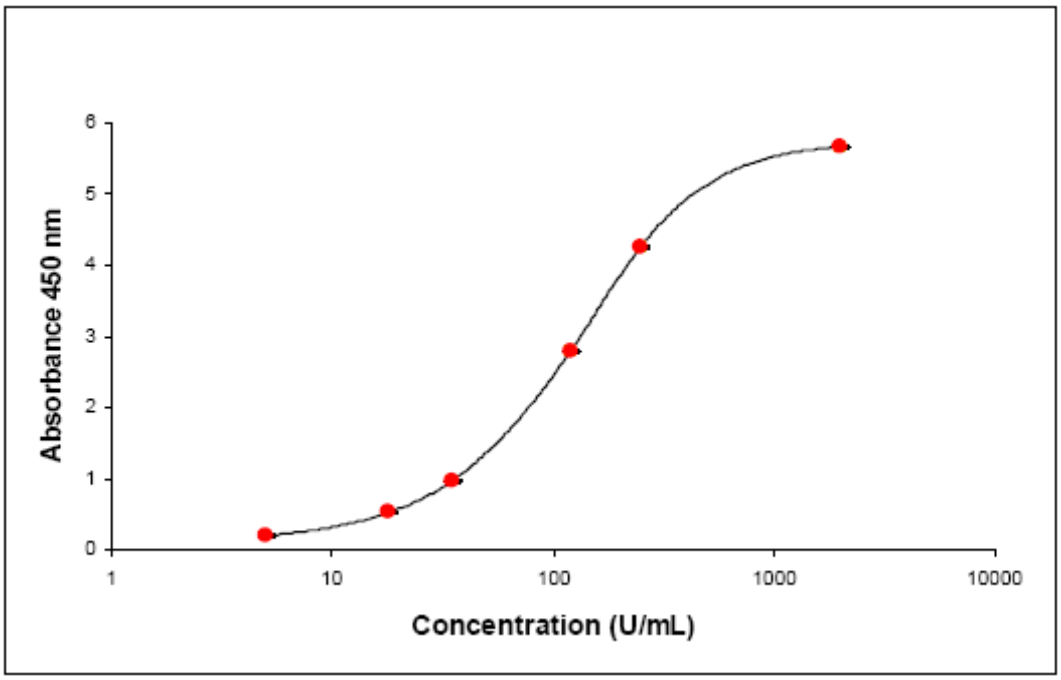
A calibration curve can be established by plotting calibrator concentration on the x-axis (log scale) against the absorbance of the calibrators on the y-axis (linear scale). The GAD autoantibody concentrations in patient sera can then be read off the calibration curve. Other data reduction systems can be used. Most test sera will have values below 250 U/mL and the 2000 U/mL calibrator need not always be included.

8 TYPICAL RESULTS

Example only, not for calculation of actual results)

Calibrator U/mL	Absorbance 450nm	405nm
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
2000	5.671	1.668
Negative Control	0.035	0.012
Positive Control	1.374	0.418

Absorbance readings at 405nm can be converted to 450nm absorbance values by multiplying by the appropriate factor.



9 ASSAY CUT OFF

U/mL	
Negative	< 5 U/mL
Positive	≥ 5.0 U/mL

10 CLINICAL EVALUATION

10.1 Clinical Specificity and Sensitivity

In the DASP 2005 study the GAD Ab ELISA kit achieved 98% (n=100) specificity and 92% (n=50) sensitivity.

10.2 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.

10.3 Inter Assay Precision

Sample	U/mL (n=20)	CV (%)
1	96.9	5.7
2	21.0	5.2

10.4 Intra Assay Precision

Sample	U/mL (n=25)	CV (%)
3	97.2	7.3
4	20.0	8.5

10.5 Clinical Accuracy

Analysis of sera from patients with autoimmune diseases other than type 1 DM disease indicated no interference from autoantibodies to:- thyroglobulin, thyroid peroxidase, TSH receptor, dsDNA or rheumatoid factor.

10.6 Interference

No interference was observed when samples were spiked with the following materials; haemoglobin up to 5 mg/mL, bilirubin up to 20 mg/dL or intralipid up to 3000 mg/dL.

The data quoted in these instructions should be used for guidance only. It is recommended that each laboratory include its own panel of control samples in the assay. Each laboratory should establish its own normal and pathological reference ranges for GAD autoantibody levels.

11 SAFETY CONSIDERATION

This kit is intended for *in vitro* use by professional persons only. Follow the instructions carefully. Observe expiry dates stated on the labels and the specified stability for reconstituted reagents. Refer to Materials Safety Data Sheet for more detailed safety information. Material of human origin used in the preparation of the kit has been tested and found non reactive for HIV1 and 2 and HCV antibodies and HBsAg but should, none the less, be handled as potentially infectious. Wash hands thoroughly if contamination has occurred and before leaving the laboratory. Sterilise all potentially contaminated waste, including test specimens before disposal. Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy but should be handled as potentially infectious. Some components contain small quantities of sodium azide as preservative. As with all kit components, avoid ingestion, inhalation, injection or contact with skin, eyes and clothing. Avoid formation of heavy metal azides in the drainage system by flushing any kit component away with copious amounts of water.

12 ASSAY PLAN

Allow all reagents and samples to reach room temperature before use	
Pipette:	25 µL Calibrators, Controls and Patient Sera
Incubate:	1 hour at room temperature on an ELISA plate shaker at 500 shakes/min
Aspirate/Decant:	Plate
Wash:	Plate three times and tap dry on absorbent material[1]
Pipette:	100 µL GAD_{65} Biotin (reconstituted) into each well
Incubate:	1 hour at room temperature on an ELISA plate shaker at 500 shakes/min
Aspirate/Decant:	Plate
Wash:	Plate three times and tap dry on absorbent material[1]
Pipette:	100 µL SA-POD (diluted 1:20) into each well
Incubate:	20 minutes at room temperature on an ELISA plate shaker at 500 shakes/min
Aspirate/Decant:	Plate
Wash:	Plate three times, rinse with pure water and tap dry on absorbent material[1]
Pipette:	100 µL TMB into each well
Incubate:	20 minutes at room temperature in the dark
Pipette:	100 µL stop solution into each well and shake for 5 seconds
Read absorbance at 450 nm and 405 nm	
[1] It is not necessary to tap dry the plates after washing when an automatic plate washer is used. Also the pure water wash can be omitted when using an automatic washer.	

13 REFERENCES

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}.
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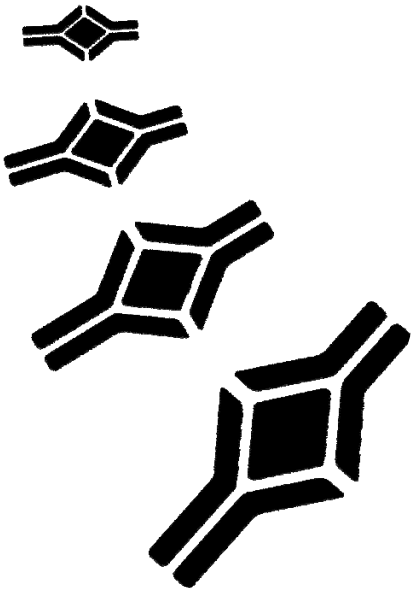

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