BioVendor Group

ENG

Instructions for Use:

GAD_{65} AUTOANTIBODY ELISA

Catalogue number:

RGDE/96R

For research use only.

BioVendor R&D

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CONTENT

HISTORY OF CHANGES

Previous version	Current version
ENG.012.A	ENG.013.A
Chapter 5., point C: see label for concentration range.	Chapter 5., point C: Concentration range is stated in the Quality Control Sheet.

1. INTENDED USE

The GAD_{65} autoantibody (GADAb) ELISA kit is intended for use by professional persons only, for the quantitative determination of GADAb 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 the islet cell antigen IA-2 or ICA-512 and zinc transporter 8 (ZnT8).

2. ASSAY PRINCIPLE

In GADAb ELISA, GADAb 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 Ab bound to the immobilised GAD_{65} on the plate. GAD_{65}-Biotin is added in a 2nd incubation step where, through the ability of GADAb in the samples to act divalently, a bridge is formed between GAD_{65} immobilised on the plate and GAD_{65}-Biotin. The amount of GAD_{65}-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 450 nm and 405 nm is then read using an ELISA plate reader. A higher absorbance indicates the presence of GAD autoantibody in the test sample. Reading at 405 nm allows quantitation of high absorbances (and should be used for concentrations of 120 U/ml or more). Low values (less than 10 U/ml) should be read off the 450 nm calibration curve. The measuring interval is 5 – 2000 U/ml (units are NIBSC 97/550).

3. STORAGE AND PREPARATION OF SERUM SAMPLES

Sera to be analyzed 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 rpm in a microfuge) to remove particulate matter. Please do not omit this centrifugation step if sera are cloudy or contain particulates.

4. MATERIAL REQUIRED BUT 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 450 nm and 405 nm.
ELISA Plate shaker, capable of 500 shakes/min (not an orbital shaker).
ELISA Plate cover.

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. Allow to stand at room temperature (20°C-25°C) for at least 30 minutes before opening.
	Ensure wells are firmly fitted into frame provided. After opening return any unused wells to the original foil packet with desiccant provided and seal with adhesive tape. Place foil bag in the self-seal plastic bag and store at 2°C-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 (Concentration range is stated in the Quality Control Sheet) 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°C – 8°C for up to 3 days after reconstitution.
F	Buffer for reconstituting GAD_{65}-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 diluting 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°C – 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 (20°C-25°C) 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 is recommended), leaving one well empty for blank (see step 12).
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 wells 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_{65}-Biotin (E) into each well (except blank). 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 SA-POD (G) into each well (except blank).
8.	Cover the frame 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 (including blank) and incubate in the dark at room temperature for 20 minutes without shaking.
11.	Pipette **100 µl** stop solution (K) to each well (including blank) cover the frame and shake for approximately 5 seconds on a plate shaker. Ensure substrate incubations are the same for each well.
12.	Within 5 minutes, read the absorbance of each well at 450 nm and 405 nm using an ELISA plate reader, blanked against the well containing **100 µl** of TMB (I) and **100 µl** stop solution (K) **only**.

7. RESULTS ANALYSIS

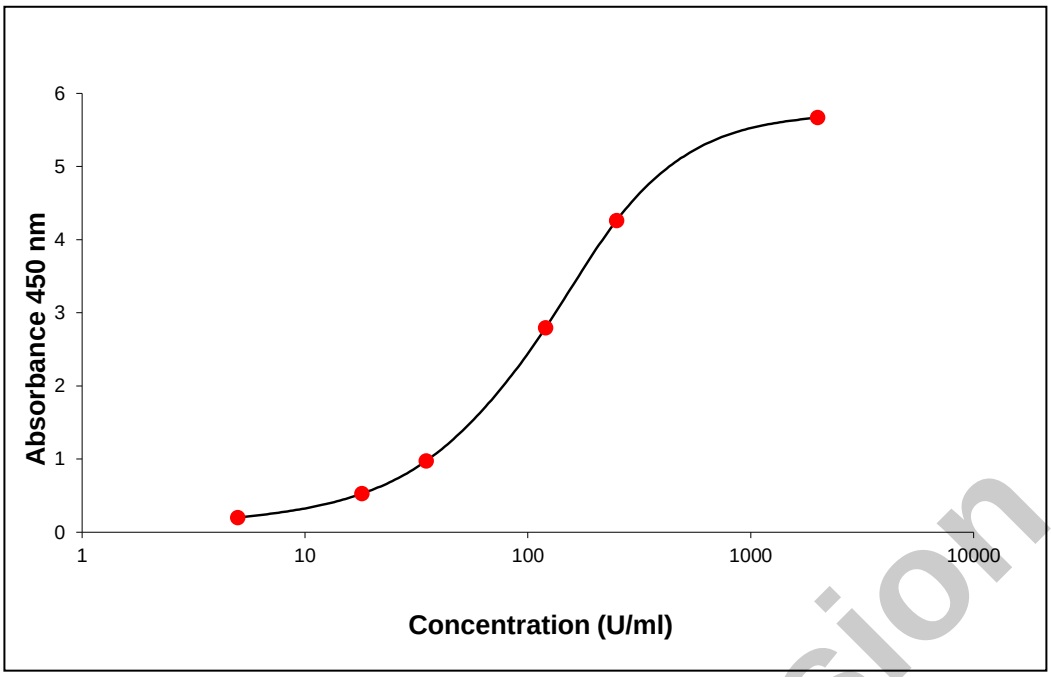
A calibration curve can be established by plotting calibrator concentration on the x-axis (log scale) against the absorbance of the calibrators on the y-axis (linear scale). The GADAb concentrations in patient sera can then be read off the calibration curve [plotted at BioVendor as a spline log/lin curve (smoothing factor = 0)]. Other data reduction systems can be used. The negative control can be assigned a value of 0.5 U/ml to assist in computer processing of assay results. Most test sera will have values below 250 U/ml and the 2000 U/ml calibrator need not always be included. Samples with high GADAb concentrations can be diluted in GADAb negative serum or the kit negative control (D). For example, 20 µl of sample plus 180 µl of diluent to give a 10x dilution. Other dilutions (e.g. 100x) can be prepared from a 10x dilution or otherwise as appropriate. Some sera will not dilute in a linear way according to the kit calibrators (standardised against NIBSC 97/550).

8. TYPICAL RESULTS

Example only, not for calculation of actual results

Calibrator U/ml	Absorbance 450 nm	Conc U/ml	Absorbance 405 nm	Conc U/ml
B1	0.199	**5**	0.061	**5**
B2	0.527	**18**	0.164	**18**
B3	0.975	**35**	0.301	**35**
B4	2.794	**120**	0.843	**120**
B5	4.264	**250**	1.254	**250**
B6	5.671	**2000**	1.668	**2000**
Negative Control (D)	0.035	**0**	0.012	**0**
Positive Control (C)	1.374	**49.2**	0.418	**49.6**

Absorbance readings at 405 nm 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 U/ml

This cut off has been validated at BioVendor. However each laboratory should establish its own normal and pathological reference ranges for GADAb levels. Also it is recommended that each laboratory include its own panel of control samples in the assay.

10. CLINICAL EVALUATION

10.1 Clinical Specificity and Sensitivity

In the DASP 2005 study the GADAb 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	97	5.7
2	21	5.2
3	5.7	6.4

10.4 Intra Assay Precision

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

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 (n=10), TSH receptor (n=20). One sample positive for dsDNA (n=10) and one sample positive for rheumatoid factor (n=30) were positive for GADAb.

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.

11. SAFETY CONSIDERATION

11.1 Streptavidin Peroxidase (SA-POD)

Signal word: Warning
Hazard statement(s)

H317: May cause an allergic skin reaction
Precautionary statement(s)

P261: Avoid breathing mist, vapours
P272: Contamined work clothing should not be allowed out of the workplace
P280: Wear protective gloves/protective clothing/ eye protection/face 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

11.2 Peroxidase Substrate (TMB)

Signal word: Danger
Hazard statement(s)

H360D: May damage the unborn child
Precautionary statement(s)

P202: Do not handle until all safety precautions have been read and understood
P280: Wear protective gloves/protective clothing/ eye protection/face 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

11.3 Diluent for SA-POD

Hazard statement(s)
EUH208: Contains 2-Chloroacetamide. May produce an allergic reaction.

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 shelf life for coated wells, reconstituted reagents and diluted 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 (except blank)
Incubate:	1 hour at room temperature on an ELISA plate shaker at 500 shakes/min
Aspirate/Decant:	ELISA Plate (A)
Wash:	ELISA Plate (A) three times and tap dry on absorbent material[1]
Pipette:	**100 µl** GAD_{65}-Biotin (reconstituted) into each well (except blank)
Incubate:	1 hour at room temperature on an ELISA plate shaker at 500 shakes/min
Aspirate/Decant:	ELISA Plate (A)
Wash:	ELISA Plate (A) three times and tap dry on absorbent material[1]
Pipette:	**100 µl** SA-POD (diluted 1:20) into each well (except blank)
Incubate:	20 minutes at room temperature on an ELISA plate shaker at 500 shakes/min
Aspirate/Decant:	ELISA Plate (A)
Wash:	ELISA Plate (A) three times, rinse with pure water and tap dry on absorbent material[1]
Pipette:	**100 µl** TMB into each well (including blank)
Incubate:	20 minutes at room temperature **in the dark (without shaking)**
Pipette:	**100 µl** stop solution into each well (including blank) and shake for 5 seconds
Read absorbance at 450 nm and 405 nm, within 5 minutes of adding stop solution.	
[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

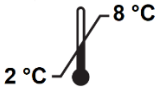
S. Chen et al
Sensitive non-isotopic assays for autoantibodies to IA2 and to a combination of both IA2 and GAD_{65}.
Clinica Chimica Acta 2005 357:74-83

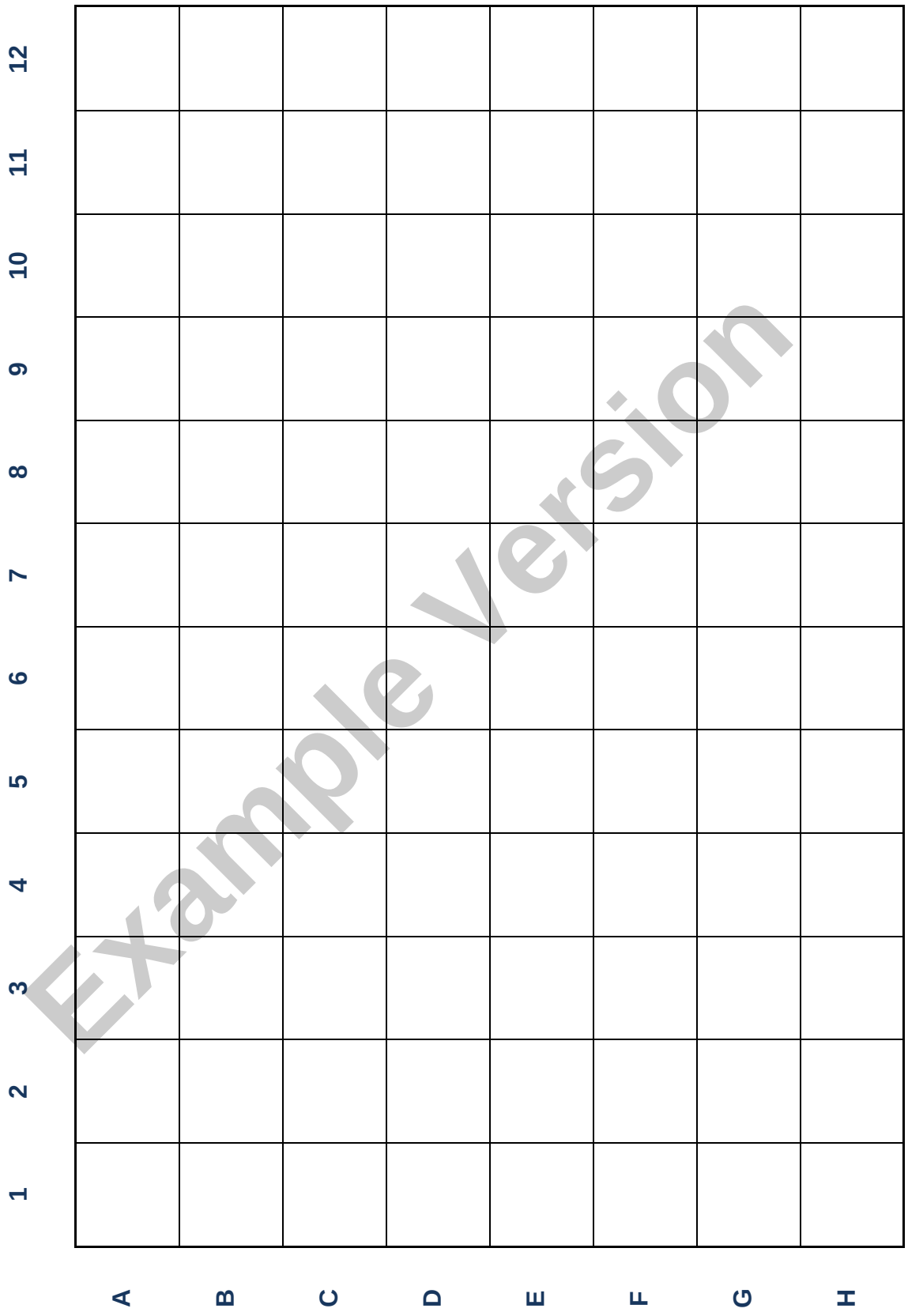
E. Nilson et al
Calcium addition to EDTA plasma eliminates falsely positive results in the RSR GADAb ELISA.
Clinica Chimica Acta 388 (2008) 130-134

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

14. EXPLANATION OF SYMBOLS

	Catalogue number
	Batch code
	Caution
	Use by date
	Temperature limit
	Manufacturer
	Read electronic instructions for use - eIFU
	The content is sufficient for 96 tests
	Biological risks



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