RSR

ElisaRSR™ GADAb

Glutamic Acid Decarboxylase (GAD) Autoantibody ELISA kit from RSR – Instructions for use

CE

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INTENDED USE

The RSR 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), the islet cell antigen IA-2 or ICA-512 and zinc transporter 8 (ZnT8).

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

PATENTS

The following patents apply:

European patent EP 1 448 993 B1, Chinese patent ZL 02822274.1, Indian patent 226484, Japanese patent 5711449 and US patents US 8,129,132 B2, US 9,435,797 B2 and US 10,481,156 B2.

ASSAY PRINCIPLE

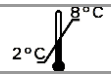
In RSR's GADAb ELISA, GADAb in patients' sera, calibrators and controls are allowed to interact with GAD_{65} coated onto ELISA plate wells. After a 1 hour incubation, the samples are discarded leaving GADAb bound to the immobilised GAD_{65} on the plate. GAD_{65}-Biotin is added in a 2nd incubation 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 GADAb the test sample. Reading at 405 nm allows quantitation of high absorbances (and should be used for concentrations of 200 u/mL or more). Low values (less than 10 u/mL) should be read off the 450 nm calibration curve. If it is possible to read at only one wavelength 405nm may be used. The measuring interval is 5 – 2000 u/mL (units are NIBSC 97/550).

STORAGE AND PREPARATION OF TEST 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, bring test sera to 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.

SYMBOLS

Symbol	Meaning
CE	EC Declaration of Conformity
IVD	In Vitro Diagnostic Device
REF	Catalogue Number
LOT	Lot Number
	Consult Instructions
	Manufactured By

	Sufficient for
	Expiry Date
	Store
CONTROL -	Negative Control
CONTROL +	Positive Control

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).
ELISA Plate cover.

PREPARATION OF REAGENTS SUPPLIED

Store unopened kit and components at 2 - 8°C

A	**GAD$_{65}$ Coated Wells** 12 breakapart strips of 8 wells (96 in total) in a frame and sealed in a foil bag. Allow to stand at room temperature (20-25 °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 bag with desiccant provided and seal with adhesive tape. Place foil bag in the self-seal plastic bag 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 to use
C	**Positive Control** **(see label for concentration range)** 0.7 mL Ready to use
D	**Negative Control** 0.7 mL Ready to 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	**Reconstitution Buffer for GAD$_{65}$-Biotin** 2 x 15 mL, coloured red Ready to 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 to use
I	**Peroxidase Substrate (TMB)** 15 mL Ready to 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 to use

ASSAY PROCEDURE

Allow all reagents to stand at room temperature (20-25 °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, 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 frame, 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 15 minutes, read the absorbance of each well at 450nm 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**.

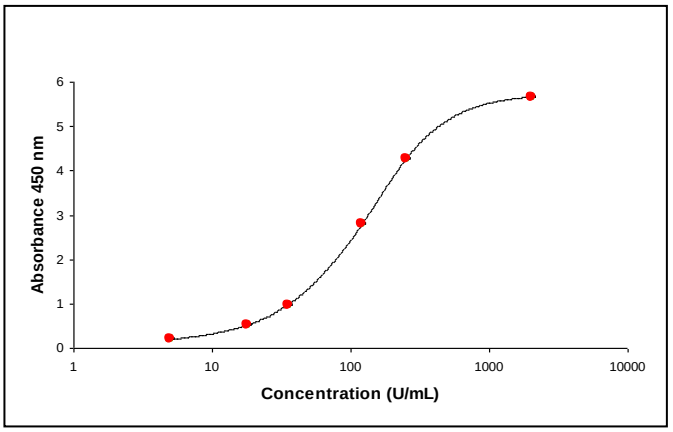
RESULT ANALYSIS

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 patients' sera can then be read off the calibration curve [plotted at RSR 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 Ab 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).

TYPICAL RESULTS (Example only, not for calculation of actual results)

Calibrator	A450 nm	Conc. u/mL	A405 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 405nm can be converted to 450nm absorbance values by multiplying by the appropriate factor (3.4 in the case of equipment used at RSR).



ASSAY CUT OFF

Cut off	u/mL
Negative	< 5 u/mL
Positive	≥ 5 u/mL

This cut off has been validated at RSR. 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.

CLINICAL EVALUATION

Clinical Specificity and Sensitivity

In the DASP 2005 study the RSR 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.

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

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 GADAb.

Interference

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

SAFETY CONSIDERATIONS

Streptavidin Peroxidase (SA-POD)

Signal word: Warning

Hazard statement(s)

H317: May cause an allergic skin reaction

Precautionary statement(s)

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

Peroxidase Substrate (TMB)

Signal word: Danger

Hazard statement(s)

H360: May damage fertility or the unborn child

Precautionary statement(s)

P280: Wear protective gloves/protective clothing/ eye protection/face protection

P308 + P313: IF exposed or concerned: Get medical advice/attention

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 and diluted reagents. Refer to 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 components away with copious amounts of water.

ASSAY PLAN

Allow all reagents and samples to reach room temperature (20-25°C) before use	
Pipette:	**25 µL** calibrators (B1-6), controls (C and D) 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 (E) (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 (G) (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 (I) into each well (including blank)
Incubate:	20 minutes at room temperature **in the dark (without shaking)**
Pipette:	**100 µL** stop solution (K) into each well (including blank) and shake for 5 seconds
Read absorbance at 450 nm and 405 nm, within 15 minutes of adding stop solution	
[1] It is not necessary to tap dry the plates after washing when an automatic plate washer is used. The pure water wash can be omitted when using an automatic washer.	