ElisaRSR™ IA-2 Ab Version 2

IA-2 Autoantibody ELISA Version 2 Kit – Instructions for use

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INTENDED USE

The RSR IA-2 autoantibody (IA-2 Ab) ELISA Version 2 kit is intended for use by professional persons only, for the quantitative determination of IA-2 Ab 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

S. Chen et al. Sensitive non isotopic assays for autoantibodies to IA-2 and to a combination of both IA-2 and GAD_{65}.
Clinica Chimica Acta (2005) 357: 74-83.

E. Nilsson et al. Calcium addition to EDTA plasma eliminates falsely positive results in the RSR GADAb ELISA.
Clinica Chimica Acta (2008) 388: 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. Torn 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 10,488,410 B2.

ASSAY PRINCIPLE

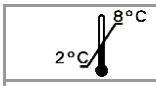
In RSR's IA-2 Ab ELISA Version 2, IA-2 Ab in patients' sera, calibrators and controls are allowed to interact with IA-2 coated onto ELISA plate wells. After a 16 – 20 hour incubation, the samples are discarded leaving IA-2 Ab bound to the IA-2 coated wells. IA-2-Biotin is added in a 2nd incubation step where, through the ability of IA-2 Ab to act divalently, a bridge is formed between the IA-2 immobilised on the plate and IA-2-Biotin. The amount of IA-2-Biotin is then determined in a third incubation step by the addition of streptavidin peroxidase (SA-POD) which binds specifically to Biotin. Excess, unbound SA-POD is then washed away and addition of the peroxidase substrate 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 from blue to yellow. The absorbance of the yellow reaction mixture at 405 nm and 450 nm is then read using an ELISA plate reader. A higher absorbance indicates the presence of IA-2 Ab 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). It is recommended that low values (less than 35 u/mL) should be read off the 450 nm calibrator curve. If it is possible to read at only one wavelength 405 nm may be used. The measuring interval is 7.5 – 4000 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. 100 µL is sufficient for one assay (duplicate 50 µL determinations). Repeated freeze thawing or increases in storage temperature must be avoided. Studies in which EDTA, citrate and heparin plasma samples were spiked with IA-2 Ab positive sera showed minor changes in signal compared with spiked serum from the same donor. In particular the absorbance values at 450 nm with spiked EDTA, citrate and heparin plasmas were 84% - 112% of spiked serum (8 samples with serum concentrations ranging from 2.3 u/mL – 505 u/mL) or 81% - 114% in terms of u/mL.
When required, bring test sera to room temperature and mix gently to ensure homogeneity. Centrifuge serum prior to assay (preferably for 5 min at about 10,000 rpm i.e. about 10,000 g 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, 50 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.

PREPARATION OF REAGENTS SUPPLIED

Store unopened kit and all components (A – M) at 2–8°C.

A	**IA-2 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 (20-25 °C) for at least 30 minutes before opening.
	Ensure that wells are firmly fitted into the frame provided. After opening return any unused wells with desiccant provided to the original foil bag 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.
B	**Negative Control** 0.7 mL Ready for use
C 1-5	**Calibrators** 7.5, 35, 120, 350, 4000 u/mL (units are NIBSC 97/550) 5 x 0.7 mL Ready for use
D	**Positive Control** **(see label for concentration range)** 0.7 mL Ready for use
E	**Reaction Enhancer** 4 mL, coloured red Ready for use
F	**Concentrated Wash Solution** 125 mL Concentrated
	Dilute 10 X with pure water before use. Store at 2–8°C up to kit expiry date.
G	**IA-2-Biotin** 3 vials Lyophilised
	Immediately before use, reconstitute with the volume indicated on the label with cold Reconstitution Buffer for IA-2-Biotin (H). When more than one vial is used, pool and mix gently before use.
H	**Reconstitution Buffer for IA-2-Biotin** 2 x 15 mL, coloured blue Ready for use
J	**Streptavidin Peroxidase (SA-POD)** 0.7 mL Concentrated
	Dilute 1 in 20 with diluent for SA-POD (K). For example, 0.5 mL (J) + 9.5 mL (K). Store at 2–8°C for up to 20 weeks after dilution.
K	**Diluent for SA-POD** 15 mL Ready for use
L	**Peroxidase Substrate (TMB)** 15 mL Ready for use
M	**Stop Solution** 12 mL Ready for use

ASSAY PROCEDURE

Allow all reagents except IA-2-Biotin (G) and Reconstitution Buffer for IA-2-Biotin (H) to stand at room temperature (20-25 °C) for at least 30 minutes before use. Do not reconstitute IA-2-Biotin until step 5 below. A repeating Eppendorf type pipette is recommended for steps 2, 5, 8, 10 & 11.

Day 1	**1.**	Pipette **50 µL** of negative control (B), calibrators (C1-5), positive control (D) and patients' sera, into respective wells (A), in duplicate, leaving one well empty for blank (see step 12).
	2.	Pipette **25 µL** of reaction enhancer (E) into each well (except blank).
	3.	Cover the frame and shake the wells for 5 seconds at 500 shakes per min then incubate overnight, without shaking, for 16-20 hours at 2–8°C.
Day 2	**4.**	Use an ELISA plate washer to aspirate and wash the plate 3 times with diluted wash solution (F). If a plate washer is not available, discard the well contents by briskly inverting the frame of wells over a suitable receptacle, wash the wells 3 times manually and finally tap the inverted wells gently on a clean dry absorbent surface.
	5.	Pipette **100 µL cold reconstituted IA-2-Biotin (G)** into each well (except blank). Avoid splashing the material out of the wells during addition.
	6.	Cover the frame, and incubate at **2-8°C** for 1 hour without shaking.
	7.	Repeat wash step 4.
	8.	Pipette **100 µL** of diluted SA-POD (J) into each well (except blank) and incubate at room temperature for 20 minutes on an ELISA plate shaker (500 shakes per min).
	9.	Repeat wash step 4. 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.

Day 2 continued	10.	Pipette **100 µL** of TMB (L) into each well (including blank) and incubate in the dark at room temperature for 20 minutes without shaking.
	11.	Pipette **100 µL** stop solution (M) into each well (including blank), cover the frame and shake for approximately 5 seconds on a plate shaker (500 shakes per min). Ensure substrate incubations are the same for each well.
	12.	Using an ELISA plate reader blanked against the well containing **100 µL** of TMB (L) and **100 µL** stop solution (M) **only**, read immediately the absorbance of each well at 405 nm and then 450 nm if using the 4000 u/mL calibrator or read within 5 minutes if this has been excluded.

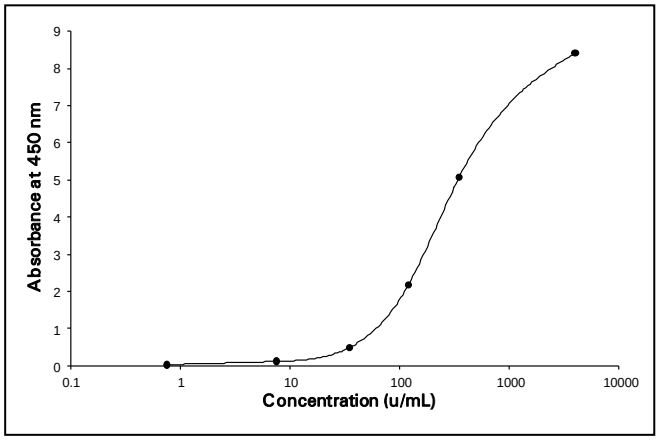
RESULT ANALYSIS

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 IA-2 Ab 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 (B) can be assigned a value of 0.75 u/mL to assist in computer processing of assay results. Many test sera will have values below 350 u/mL and the 4000 u/mL calibrator need not always be included. Samples with high IA-2 Ab concentrations can be diluted in kit negative control (B). For example, 15 µL of sample plus 135 µL of negative control 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.

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

	A450 nm	Conc. u/mL	A405 nm	Conc. u/mL
Negative Control (B)	0.027	0	0.013	0
C1	0.116	7.5	0.041	7.5
C2	0.494	35	0.148	35
C3	2.170	120	0.649	120
C4	5.083	350	1.495	350
C5	8.408	4000	2.473	4000
Positive Control (D)	2.198	122	0.655	121

For absorbance readings at 450 nm above 3.0, the absorbance readings at 405 nm can be converted to 450 nm absorbances by multiplying by the appropriate factor (3.4 in the case of equipment used at RSR).



ASSAY CUT OFF

Negative	< 7.5 u/mL
Positive	≥ 7.5 u/mL

This cut off has been validated at RSR. However, each laboratory should establish its own normal and pathological reference ranges for IA-2 Ab 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 IASP 2016 study the RSR IA-2 Ab ELISA Version 2 kit showed 98% (n = 90) specificity and 76% (n = 50) sensitivity.

Sera from 533 individual healthy blood donors were tested in the RSR IA-2 Ab ELISA Version 2 kit. 523 (98.1%) sera were identified as being negative (less than the cut off of 7.5 u/mL) for IA-2 Ab.

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 0.95 u/mL.

Inter Assay Precision

Sample	u/mL (n = 20)	CV (%)
1	41	4.5
2	140	4.2

Intra Assay Precision

Sample	u/mL (n = 25)	CV (%)
A	12	3.1
B	41	1.3
C	80	1.6
D	296	2.1
E	6.1	2.1

Clinical Accuracy

Analysis of sera from patients with autoimmune diseases other than type 1 DM indicated no interference from autoantibodies to; TSH receptor (n = 19). 5% (n = 20) of sera positive for autoantibodies to thyroglobulin and thyroid peroxidase and 5% (n = 20) of sera positive for rheumatoid factor were positive for IA-2 Ab using the RSR IA-2 Ab ELISA Version 2. These sera were also positive in the RSR GADAb ELISA.

Interference
No interference was observed when samples were spiked with the following materials; haemoglobin at 5 mg/mL or bilirubin at 20 mg/dL. Interference was observed with intralipid at 1000 and 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, diluted or reconstituted 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. These materials should be handled as potentially infectious. Some components contain small quantities of sodium azide as preservative. With all kit components, avoid ingestion, inhalation, injection and 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.

ASSAY PLAN

Day 1	Allow all reagents and samples except IA-2-Biotin (G) and Reconstitution Buffer for IA-2-Biotin (H) to reach room temperature (20 - 25°C) before use	
	Pipette:	**50 µL** negative control (B), calibrators (C1 – 5), positive control (D) and patient sera (except blank)
	Pipette:	**25 µL** reaction enhancer (E) (except blank)
	Mix:	Shake on an ELISA plate shaker at 500 shakes/min for 5 seconds
	Incubate:	Overnight (16–20 hours) at 2–8°C, without shaking
Day 2	Aspirate/Decant:	ELISA plate (A)
	Wash:	ELISA plate (A) three times (dry on absorbent material for manual wash)
	Pipette:	**100 µL cold** IA-2-Biotin (G) (reconstituted with **cold** Reconstitution Buffer (H)) into each well (except blank)
	Incubate:	1 hour at **2-8 °C, without shaking**
	Aspirate/Decant:	ELISA plate (A)
	Wash:	ELISA plate (A) three times (dry on absorbent material for manual wash)
	Pipette:	**100 µL** SA-POD (J) (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, (additional rinse with pure water and dry on absorbent material for manual wash)
	Pipette:	**100 µL** TMB (L) into each well (including blank)
	Incubate:	20 minutes at room temperature **in the dark (without shaking)**
	Pipette:	**100 µL** stop solution (M) into each well (including blank) and shake for 5 seconds
	Read absorbance immediately at 405 nm and then 450 nm if using the 4000 u/mL calibrator or within 5 minutes if it is excluded.	
	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 from the final wash step when using an automatic washer.	