ElisaRSR™ Fast IA-2 Ab

Fast IA-2 Autoantibody ELISA Kit Instructions for use

CE

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INTENDED USE

The RSR Fast IA-2 autoantibody (IA-2 Ab) ELISA 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 (T1D). 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.

PATENTS

The following patents apply:
US patents US 8,129,132 B2 and US 10,488,410 B2.

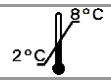
ASSAY PRINCIPLE

The RSR Fast IA-2 Ab ELISA is an enzyme immunoassay which uses the ability of IA-2 antibodies to act divalently and form a bridge between immobilized IA-2 on the plate and liquid-phase IA-2-Biotin.
IA-2 Ab in patients' sera, calibrators and controls are allowed to interact with IA-2 coated onto ELISA plate wells. After a 2 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 a bridge is formed between the IA-2 immobilised on the plate, the antibody and IA-2-Biotin. Unbound IA-2-Biotin is then removed in a wash step. The amount of IA-2-Biotin is then determined in a 3rd incubation step by the addition of streptavidin peroxidase (SA-POD) which binds specifically to Biotin. Excess SA-POD is washed away and the addition of a colorogenic peroxidase substrate 3,3',5,5' – tetramethylbenzidine (TMB) results in a blue colour. This reaction is stopped by the 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 when the OD at 450 nm is greater than 3.0. It is recommended that low values (less than 35 u/mL) should be read off the 450 nm calibrator curve. If it is only possible to read at one wavelength, 405 nm may be used. The measuring interval is 7.5 – 4000 u/mL (units are NIBSC 97/550).
This Fast IA-2 Ab ELISA kit assay is performed within 4 hours and without refrigeration and may be particularly suitable for automated ELISA processors.

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 (RSR recommends duplicate 50 µL determinations). Repeated freeze thawing or increases in storage temperature must be avoided.
Do not use lipaemic or haemolysed serum samples.
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
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
C1-5	**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	**IA-2-Biotin** 3 vials Lyophilised
	Immediately before use, reconstitute each vial with the volume indicated on the label with Reconstitution Buffer for IA-2-Biotin (G). When more than one vial is used, pool and mix gently before use.
G	**Reconstitution Buffer for IA-2-Biotin** 2 x 15 mL, coloured blue Ready for use
H	**Streptavidin Peroxidase (SA-POD)** 0.7 mL Concentrated
	Dilute 1 in 20 with diluent for SA-POD (J). For example, 0.5 mL (H) + 9.5 mL (J). Store at 2–8°C for up to 20 weeks after dilution.
J	**Diluent for SA-POD** 15 mL Ready for use
K	**Peroxidase Substrate (TMB)** 15 mL Ready for use
L	**Stop Solution** 12 mL Ready for use
M	**Concentrated Wash Solution** 125 mL Concentrated
	Dilute 10 X with pure water before use. Store at 2–8°C up to kit expiry date.

ASSAY PROCEDURE

Allow all reagents 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. All assay steps should be performed at room temperature (20-25 °C).

1.	Pipette **50 µL** of negative control (B), calibrators (C1-5), positive control (D) and patients' sera, into respective wells (A), (in duplicate is recommended), leaving two wells 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 2 hours on an ELISA plate shaker (500 shakes per min).
4.	Use an ELISA plate washer to aspirate and wash the plate 3 times with diluted wash solution (M). 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** reconstituted IA-2-Biotin (F) into each well (except blank). Avoid splashing the material out of the wells during addition.
6.	Cover the frame, and incubate for 1 hour on an ELISA plate shaker (500 shakes per min).
7.	Repeat wash step 4.
8.	Pipette **100 µL** of diluted SA-POD (H) into each well (except blank) and incubate 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.
10.	Pipette **100 µL** of TMB (K) into each well (including blank) and incubate in the dark for 20 minutes without shaking.
11.	Pipette **100 µL** stop solution (L) into each well (including blank), cover the frame and shake for approximately 5 seconds on a plate shaker (500 shakes per minute). Ensure substrate incubations are the same for each well.
12.	Using an ELISA plate reader blanked against the well containing **100 µL** of TMB (K) and **100 µL** stop solution (L) **only**, read immediately the absorbance of each well at 405 nm and then 450 nm.

RESULT ANALYSIS

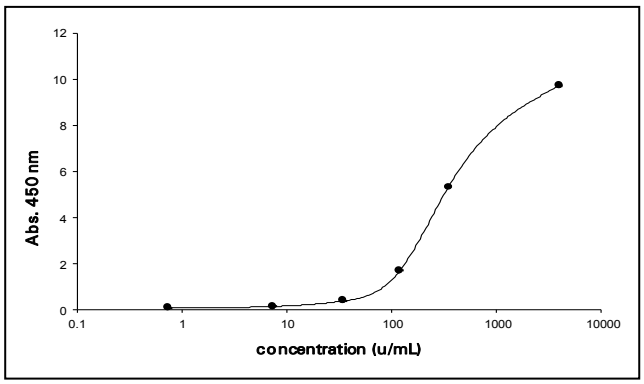
A calibration curve can be established by plotting calibrator concentration on the x-axis (log scale) against the absorbance of the calibrators on the y-axis (linear scale). The IA-2 Ab concentrations in patients' sera can then be read off the calibration curve [plotted at RSR as a spline log/lin curve (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 may be excluded.

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)	0.062	0	0.017	0
C1	0.132	7.5	0.040	7.5
C2	0.371	35	0.116	35
C3	1.686	120	0.532	120
C4	5.338	350	1.570	350
C5	9.717	4000	2.858	4000
Positive Control (D)	1.804	126	0.569	126

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

48 out of 50 (96%) serum samples from patients diagnosed with T1D were positive for IA-2 Abs in the Fast IA-2 Ab ELISA (assay sensitivity 96%). Sera from 155 individual healthy blood donors were tested in the RSR Fast IA-2 Ab ELISA kit and 155 (100%) sera were identified as being negative.

Limit of Blank and Limit of Detection

The kit negative control and a low analyte sample were assayed 20 times in 3 different kit lots and the mean limit of blank and limit of detection calculated.

Limit of Blank at 2 standard deviations was 2.08 u/mL.
Limit of Detection was 2.74 u/mL.

Clinical Accuracy

One out of 14 sera from patients with Graves' disease (7.1%) and one out of 20 with rheumatoid arthritis (5%) were positive in the Fast IA-2 Ab ELISA. Two out 25 from type 2 diabetes patients (4%) were also positive.

Inter Assay Precision

Sample	u/mL (n = 20)	CV (%)
A	13	10.5
B	36	8.0
C	76	4.4
D	131	4.6
E	485	6.4

Intra Assay Precision

Sample	u/mL (n = 25)	CV (%)
1	14	6.5
2	40	3.4
3	78	2.3
4	140	3.6
5	521	4.6

SAFETY CONSIDERATIONS

Streptavidin Peroxidase (SA-POD) and Reaction Enhancer

Signal word: Warning

Hazard statement(s)

H317: May cause an allergic skin reaction

Precautionary statement(s)

P261: Avoid breathing mist, vapours
P272: Contaminated 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

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

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

Allow all reagents and samples 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) into ELISA plate wells (A)
Pipette:	**25 µL** reaction enhancer (E) (except blank)
Incubate:	2 hours at room temperature on an ELISA plate shaker at 500 shakes/min
Aspirate/Decant:	ELISA plate (A)
Wash:	ELISA plate (A) three times (dry on absorbent material for manual wash)
Pipette:	**100 µL** IA-2-Biotin (F) (reconstituted with Reconstitution Buffer (G)) 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 (dry on absorbent material for manual wash)
Pipette:	**100 µL** SA-POD (H) (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 (K) into each well (including blank)
Incubate:	20 minutes at room temperature **in the dark (without shaking)**
Pipette:	**100 µL** Stop Solution (L) into each well (including blank) and shake for 5 seconds
Read absorbance immediately at 405 nm and then 450 nm	
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 or an automatic ELISA processor.	