ElisaRSR™ 3 Screen ICA™

3 Screen Islet Cell Autoantibody ELISA Kit - Instructions for use

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RSR Limited
Parc Ty Glas, Llanishen,
Cardiff CF14 5DU United Kingdom
Tel.: +44 29 2068 9299 Fax: +44 29 2075 7770
Email: info@rsrltd.com Website: www.rsrltd.com

INTENDED USE

The RSR 3 Screen Islet Cell Autoantibody (3 Screen) ELISA kit is intended for use by professional persons only, for quantitative determination of GAD, IA-2 and ZnT8 autoantibodies (Ab) in human serum. Ab 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 named IA-2 or ICA-512 and zinc transporter 8 (ZnT8). RSR's 3 Screen ELISA allows simultaneous measurement of GAD, IA-2 and ZnT8 Ab in the same sample.

REFERENCES

M. Amoroso et al
"3 Screen islet cell autoantibody ELISA: A sensitive and specific ELISA for the combined measurement of autoantibodies to GAD_{65}, to IA-2 and to ZnT8."
Clin. Chim. Acta. 2016 462:60 – 64

A. G. Ziegler et al
"3 Screen ELISA for high-throughput detection of beta cell autoantibodies in capillary blood."
Diabetes Technol. Ther. 2016 18:687 – 693

PATENTS

The following patents apply:

European patents EP 1 448 993 B1, EP 1 563 071 B1 and EP 2 118 309 B1, Chinese patents ZL 02822274.1, CN 1738900 B and ZL 200780051859.3, Indian patents 226484 and 279741, Japanese patents 5711449, 4498144 and 5694668 and US patents US 8,129,132 B2, US 9,435,797 B2, US 7,851,164 B2, US 9,023,984 B2, US 6,682,906 B1 and US 10,481,156 B2.

ASSAY PRINCIPLE

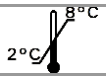
In RSR's 3 Screen ELISA, GAD, IA-2 and ZnT8 Ab in patients' sera, reference preparation or calibrators (optional) and controls are allowed to interact with GAD_{65}, IA-2 and ZnT8 coated onto ELISA plate wells. After a 16 - 20 hour incubation, the samples are discarded leaving any GAD, IA-2 and/or ZnT8 Ab in the patient sera, reference preparation or calibrators (optional) and controls bound to the GAD_{65}, IA-2 and ZnT8 coated wells. A mixture of GAD_{65}-Biotin, IA-2-Biotin and ZnT8-Biotin is then added and during a 2nd incubation step where, through the ability of GAD, IA-2 and ZnT8 Ab to act divalently, a bridge is formed between the GAD_{65}, IA-2 or ZnT8 immobilised on the plate and GAD_{65}-Biotin, IA-2-Biotin and ZnT8-Biotin respectively. Unbound GAD_{65}/IA-2/ZnT8-Biotin is then removed in a wash step and the amount of bound GAD_{65}/IA-2/ZnT8-Biotin determined (in a 3rd incubation step) by 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'-tetramethyl-benzidine (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, IA-2 and/or ZnT8 Ab in the test sample. Reading at 405nm allows quantitation of high absorbances. It is recommended that low absorbance values are measured at 450nm. If it is possible to read at only one wavelength 405nm may be used.

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 (RSR recommends 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 sera 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 25μL and 100μL.
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 kits and all components (A-M) at 2–8°C.

A	**3 Screen 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 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 3 months.
B	**Negative Control** 0.3 mL Ready for use
C1	**GADAb Positive Control** 0.3 mL Ready for use
C2	**IA-2 Ab Positive Control** 0.3 mL Ready for use
C3	**ZnT8 Ab Positive Control** 0.3 mL Ready for use
D	**Reference Preparation** 0.3 mL Ready for use
E1-5	**Calibrators (optional)** 5, 15, 100, 400 and 2000 u/mL (units are arbitrary RSR units) 5 x 0.3 mL 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.
G	**3 Screen-Biotin** 3 vials Lyophilised
	Immediately before use reconstitute each vial with 5.5 mL of reconstitution buffer for 3 Screen-Biotin (H). When more than one vial is used, pool and mix gently before use.
H	**Reconstitution Buffer for 3 Screen-Biotin** 2 x 15 mL Coloured red Ready for use
J	**Streptavidin Peroxidase (SA-POD)** 0.7 mL Concentrated
	Dilute 1 in 20 with diluent for SA-POD (K). For example, 0.5mL (J) + 9.5mL (K). Store at 2–8°C for up to 28 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 and test sera to stand at room temperature (20-25°C) for at least 30 minutes before use except 3 Screen-Biotin and reconstitution buffer for 3 Screen-Biotin. A repeating Eppendorf type pipette is recommended for steps 4, 7, 10 and 11.

Day 1	**1.**	Pipette **25 µL** of negative control (B), positive controls (C1–3), reference preparation (D) or (if used) calibrators (E1-5) and patients' sera into respective wells (A), (in duplicate is recommended), leaving one well empty for blank (see step 12).
	2.	Cover the frame and shake for approximately 5 seconds on an ELISA plate shaker (500 shakes per min) and incubate at 2–8°C (without shaking) overnight (16-20 hours)
Day 2	**3.**	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.
	4.	Pipette **100 µL** of **cold reconstituted 3 Screen-Biotin (G)** into each well (except blank). Avoid splashing the material out of the wells during addition.
	5.	Cover the frame, and incubate at 2-8°C for 1 hour (without shaking).
	6.	Repeat wash step 3.
	7.	Pipette **100 µL** of diluted SA-POD (J) into each well (except blank).

Day 2 continued	8.	Cover the frame and incubate at room temperature (20-25°C) 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 (L) into each well (including blank) and incubate in the dark at room temperature (20-25°C) 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.	Within 10 minutes, read the absorbance of each well at 405nm and then 450 nm using an ELISA plate reader, blanked against a well containing **100 µL** of TMB (L) and **100 µL** stop solution (M) only.

RESULT ANALYSIS

Calculation of results without calibrators

Index Calculation

The index values are calculated as follows:

$$\text{Index} = \frac{\text{test sample absorbance at 450nm}}{\text{reference preparation absorbance at 450nm}} \times 100$$

The index value can also be calculated using absorbance data at 405nm

97% of 1200 healthy adult male blood donor sera gave index values of less than 30 suggesting that index values of 30 or more could be considered positive in this group (see page 4).

TYPICAL RESULTS (Example only; not to be used for calculation of actual results)

	A450 nm	Index value	A405 nm	Index value
Reference Preparation (D)	0.702	100	0.222	100
Negative Control (B)	0.030	4.3	0.009	4.1
Positive Control (C1)	1.300	185	0.412	186
Positive Control (C2)	0.387	55	0.123	55
Positive Control (C3)	0.181	26	0.057	26

ASSAY INDEX VALUE CUT OFF

Negative	< 30
Positive	≥ 30

Calculation of results with calibrators

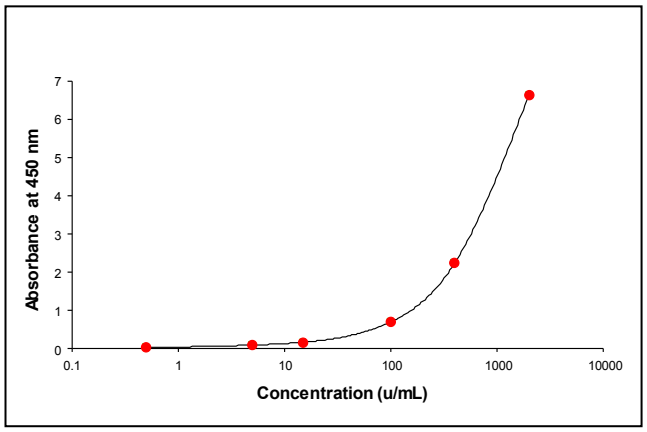
A calibration curve can be established by plotting calibrator concentration on the x-axis (log scale) against the absorbance of the calibrators on the y-axis (linear scale). The GAD, IA-2 and/or ZnT8 Ab concentrations in patients' sera can then be read off the calibration curve [plotted at RSR as a spline log/lin curve (smoothing factor = 0)]. Other data reduction methods can be used. The negative control (B) has a concentration of 0 u/mL, but can be assigned a value of 0.5 u/mL to facilitate computer processing of data.

TYPICAL RESULTS (Example only; not to be used for calculation of actual results)

	A450 nm	Conc. u/mL	A405 nm	Conc. u/mL
Negative Control (B)	0.030	0	0.009	0
E1	0.094	5	0.030	5
E2	0.167	15	0.052	15
E3	0.702	100	0.222	100
E4	2.233	400	0.711	400
E5	6.637*	2000	1.952	2000
Positive Control (C1)	1.300	213	0.412	206
Positive Control (C2)	0.387	48	0.123	51
Positive Control (C3)	0.181	17	0.057	17

*derived from 405nm value

For absorbance readings at 450nm above 3.0, the absorbance reading 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).



Samples with high GAD, IA-2 and/or ZnT8 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.

ASSAY CONCENTRATION CUT OFF

Negative	< 20 u/mL
Positive	≥ 20 u/mL

This cut off and the cut off based on index value has been validated at RSR. However each laboratory should establish its own normal and pathological reference ranges for 3 Screen. Also it is recommended that each laboratory include its own panel of control samples in the assay.

CLINICAL EVALUATION

Clinical Specificity and Sensitivity

In an analysis of 1200 healthy adult male blood donor sera 1166 (97%) gave index values of less than 30. An index of 30 was equivalent to 20 u/mL. Out of the 34 sera giving index values of 30 or greater, 33 (97%) were positive in individual assays for GADAb and/or IA-2 Ab and/or ZnT8 Ab.

Analysis of sera from 147 patients with type 1 DM (mostly with longstanding disease) indicated that 126 (86%) gave 3 Screen index values of 30 or more. There was good agreement between 3 Screen results and individual assays for GADAb and/or IA-2 Ab and/or ZnT8 Ab (concordance 94%).

Lower Detection Limit

The negative control was assayed 20 times and the mean and standard deviation calculated. The lower detection limit at +2 standard deviations was 1.3 u/mL, the index value was 8.3.

Intra Assay Precision

Sample	Mean u/mL (n = 25)	CV (%)	Mean index (n = 25)	CV (%)
1	23	7.9	32	4.0
2	25	4.5	33	2.5
3	38	5.7	42	4.4
4	145	4.6	140	4.1
5	405	4.4	336	3.4

Inter Assay Precision

Sample	Mean u/mL (n = 20)	CV (%)	Mean index (n = 20)	CV (%)
A	71	5.8	72	3.2
B	95	5.1	93	3.0
C	121	4.7	114	3.1
D	192	4.1	167	3.6
E	260	4.8	212	3.5
F	489	3.3	334	2.5
G	1158	3.3	553	3.1

Clinical Accuracy

Out of 108 sera with Graves' disease, 6 (5.6%) were 3 Screen positive (index ≥ 30). 5 of the 6 were also positive for GADAb and/or IA-2 Ab and/or ZnT8 Ab in individual Ab assays.

In the case of Addison's disease, 3 out of 10 (30%) of patients were 3 Screen positive (index ≥ 30) as were 3 out of 29 (10%) coeliac disease sera and 1 out of 20 (5%) sera from patients with rheumatoid arthritis. All 3 Screen positive sera in these 3 patient groups were also positive for GADAb and/or IA-2 Ab and/or ZnT8 Ab in individual Ab assays.

Interference

No interference was observed when samples were spiked with the following materials: haemoglobin at 500 mg/dL, bilirubin at 20 mg/dL, Intralipid up to 3000 mg/dL or Biotin at 14 μg/mL.

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

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 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 (except 3 Screen-Biotin and reconstitution buffer for 3 Screen-Biotin) and test sera to reach room temperature (20-25°C) before use		
Day 1	Pipette:	**25 µL** negative and positive controls (B and C1-3), reference preparation (D) **or** calibrators (if used E1-5) and test sera into ELISA plate (A) (except blank)
	Mix:	Shake for 5 seconds at 500 shakes/min
	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 (F))
	Pipette:	**100 µL cold** 3 Screen-Biotin (G) (reconstituted with (H)) into each well (A) (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 (F))
	Pipette:	**100 µL** SA-POD (J) (diluted 1:20) into each well (except blank)
	Incubate:	20 minutes at room temperature with shaking 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 (F))
	Pipette:	**100 µL** TMB (L) into each well (A) (including blank)
	Incubate:	20 minutes at room temperature **in the dark (without shaking)**
	Pipette:	**100 µL** stop solution (M) into each well (including blank (A)) and shake for 5 seconds
	Read absorbance at 405nm and 450nm within 10 minutes of addition of stop solution	