ElisaRSR™ Fast ZnT8 Ab™

Fast Zinc Transporter 8 (ZnT8) Autoantibody ELISA Kit – Instructions for use

CE

RSR Limited

Parc Ty Glas, Llanishen, Cardiff
CF14 5DU United Kingdom
Tel.: +44 29 20689299 Fax: +44 29 20757770
Email: info@rsrltd.com Website: www.rsrltd.com

INTENDED USE

The RSR Fast ZnT8 autoantibody (ZnT8 Ab) ELISA kit is intended for use by professional persons only, for the quantitative determination of ZnT8 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). ZnT8 Ab are directed principally to the C terminal domain of ZnT8 (residues 268 – 369). Human population gene polymorphism at the codon for the 325th amino acid results in the expression of three protein variants: Arginine (R) 325, Tryptophan (W) 325 and very rarely Glutamine (Q) 325. ZnT8 Ab may be specific to the R 325 or W 325 variant, or may be residue 325 non-specific. Sera that react with the Q allele only are extremely rare. RSR's ZnT8 Ab ELISA is capable of detecting, and quantifying, autoantibodies specific to R 325 or to W 325, or to residue 325 non-specific variants.

REFERENCES

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"Autoantibodies to zinc transporter 8 and *SLC30A8* genotype stratify type 1 diabetes risk."
Diabetologia 2009 52:1881-1888

J. M. Wenzlau et al
"Kinetics of the post-onset decline in zinc transporter 8 autoantibodies in type 1 diabetic human subjects."
J Clin Endocrinol Metab 2010 95:4712 - 4719

L. Petruzelkova et al
"The dynamic changes of zinc transporter 8 autoantibodies in Czech children for the onset of type 1 diabetes mellitus."
Diabet Med 2014 31:165 - 71

G. Dunseath et al
"Bridging-type enzyme-linked immunoassay for zinc transporter 8 autoantibody measurements in adult patients with diabetes mellitus."
Clin. Chim. Acta. 2015 447:90 - 95

PATENTS

The following patents apply:
European patents EP 1 563 071 B1 and EP 2 118 309 B1, US patents US 7,851,164 B2 and US 9,023,984 B2, Chinese patents CN 1738900 B and ZL 200780051859.3, Indian patent 279741 and Japanese patents 4498144 and 5694668.

ASSAY PRINCIPLE

In RSR's Fast ZnT8 Ab ELISA, ZnT8 Ab in test patients' sera, calibrators and controls are allowed to interact with ZnT8 coated onto ELISA plate wells. After a 2 hour incubation, the samples are discarded leaving ZnT8 Ab bound to the ZnT8 coated wells. ZnT8-Biotin is added in a 2nd incubation step where, through the ability of ZnT8 Ab in the samples to act bivalently (or polyvalently), a bridge is formed between ZnT8 bound to the wells and ZnT8-Biotin. Unbound ZnT8-Biotin is then removed in a wash step and the amount of bound 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 405 nm and 450 nm is then read using an ELISA plate reader. A higher absorbance indicates the presence of ZnT8 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. If it is possible to read at only one wavelength 405nm may be used. The measuring interval is 10 – 2000 u/mL (arbitrary RSR units).

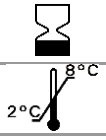
This Fast ZnT8 Ab ELISA kit assay is performed in about 4 hours and without refrigeration. It may be particularly suitable for users with automated ELISA processors.

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. 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. When required, bring test sera to room temperature and mix gently to ensure homogeneity. Centrifuge sera prior to assay (preferably for 5 min at 10-15,000 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 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 kits and all components at 2–8°C.

A	**ZnT8 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 1 month.
B1-5	**Calibrators** 10, 20, 75, 500, 2000 u/mL (units are arbitrary RSR units) 5 x 0.7 mL Ready for use
C1-2	**Positive Controls I & II** **(see label for concentration range)** 2 x 0.7 mL Ready for use
D	**Negative Control** 0.7 mL Ready for use
E	**ZnT8-Biotin** 3 vials Lyophilised
	Reconstitute each vial with 5.5 mL reconstitution buffer for ZnT8-Biotin (F). When more than one vial is used, pool the vials and mix gently before use. Store at 2–8°C for up 3 days after reconstitution.
F	**Reconstitution Buffer for ZnT8-Biotin** 2 x 15 mL Coloured red Ready for use
G	**Streptavidin Peroxidase (SA-POD)** 0.7 mL Concentrated
	Dilute 1 in 20 with Diluent for SA-POD (H) before use. 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 for use
J	**Peroxidase Substrate (TMB)** 15 mL Ready for use
K	**Stop Solution** 12 mL Ready for use
L	**Concentrated Wash Solution** 125 mL Concentrate
	Dilute 10 X with pure water before use. For example, 100 mL (L) + 900 mL pure water. Store at 2–8°C for up to kit expiry date.

ASSAY PROCEDURE

On the day of the assay 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 calibrators (B1-5), controls (C1-2 and D) and patients' sera into respective wells, in duplicate, leaving two wells empty for blanks (see step 12).
2.	Cover the frame and incubate at room temperature for 2 hours 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 (L). 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 ZnT8-Biotin (E)** into each well (except blanks). 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 blanks).
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 (J) into each well (including blanks) and incubate in the dark at room temperature for 20 minutes without shaking.
11.	Pipette **100 µL** stop solution (K) to each well (including blanks) 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 405nm, then at 450 nm, using an ELISA plate reader, blanked against the wells containing **100 µL** of TMB (J) and **100 µL** stop solution (K) **only**.

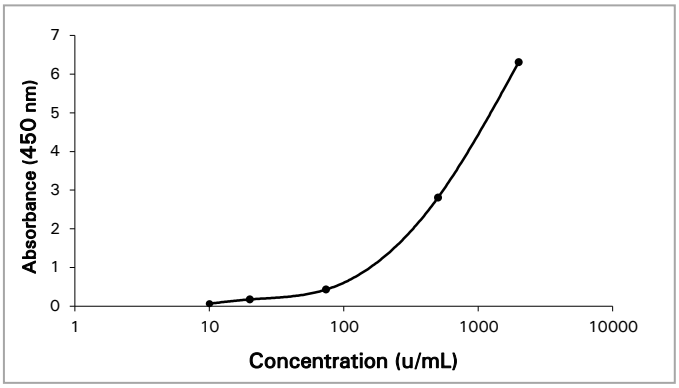
RESULT ANALYSIS

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 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 systems can be used. The negative control (D) has a concentration of 0 u/mL, but can be assigned a value of 1 u/mL to facilitate computer processing of data. Samples with high ZnT8 Ab concentrations can be diluted in kit negative control (D). 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)

Calibrator	A450 nm	Conc. u/mL	A405 nm	Conc. u/mL
B1	0.063	10	0.020	10
B2	0.172	20	0.053	20
B3	0.436	75	0.130	75
B4	2.803	500	0.864	500
B5	6.314	2000	1.825	2000
Negative Control (D)	0.012	0	0.005	0
Positive Control (CI)	0.406	70	0.123	72
Positive Control (CII)	1.194	187	0.351	174

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).



ASSAY CUT OFF

Cut off	u/mL
Negative	< 10 u/mL
Positive	≥ 10 u/mL

This cut off has been validated at RSR. However, each laboratory should establish its own normal and pathological reference ranges for ZnT8 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

Sera from 640 individual healthy blood donors were assayed in the Fast ZnT8 Ab ELISA. 631 (98.6%) were identified as being negative for ZnT8 Ab.

For IASP 2016 samples, sensitivity was 72% (n = 50) and specificity was 97% (n = 90).

Lower Detection Limit

The negative control assigned as 1.0 u/mL was assayed 20 times and the mean and standard deviation calculated. The lower detection limit at +2 standard deviations was 5.0 u/mL with standard laboratory equipment and was 2.4 u/mL for an assay performed on a Dynex DS2 automated ELISA processor.

Inter Assay Precision

Sample	Mean u/mL (n = 20)	CV (%)
A	26.3	10.1
B	52.4	12.7
C	208.7	6.2
D	403.6	8.4

Intra Assay Precision

Sample	Mean u/mL (n = 25)	CV (%)
1	20.8	4.4
2	31.0	5.7
3	208.6	2.6
4	357.0	6.5

Clinical Accuracy

Sera containing rheumatoid factor (n = 20), sera containing antibodies to the TSH receptor (n = 81) and 40 Type 2 Diabetes mellitus patients' sera were all negative for ZnT8 Ab. 15 out of 39 sera from Latent Autoimmune Diabetes of Adults (LADA) patients were positive for ZnT8 Ab.

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)

H360D: May damage 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 use by professional persons only. Follow the instructions carefully. Observe expiry dates stated on the labels and the specified stability for reconstituted reagents. Refer to the Safety Data Sheet for more detailed safety information. Avoid all actions likely to lead to ingestion. Avoid contact with skin and clothing. Wear protective clothing. 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 or contact with skin, eyes or 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) on day of use	
Pipette:	**25 µL** Calibrators, controls and patient sera into wells (except blanks)
Incubate:	2 hours at room temperature (20-25°C) on an ELISA plate shaker at 500 shakes/min
Aspirate/Decant:	Plate
Wash:	Plate three times and tap dry on absorbent material[1]
Pipette:	**100 µL** ZnT8-Biotin (reconstituted) into each well (except blanks)
Incubate:	1 hour at room temperature (20-25°C) on an ELISA plate shaker at 500 shakes/min
Aspirate/Decant:	Plate
Wash:	Plate three times and tap dry on absorbent material[1]
Pipette:	**100 µL** SA-POD (diluted 1:20) into each well (except blanks)
Incubate:	20 minutes at room temperature (20-25°C) on an ELISA plate shaker at 500 shakes/min
Aspirate/Decant:	Plate
Wash:	Plate three times, rinse with pure water and tap dry on absorbent material[1]
Pipette:	**100 µL** TMB into each well (including blanks)
Incubate:	20 minutes at room temperature (20-25°C) **in the dark**
Pipette:	**100 µL** Stop Solution into each well (including blanks) and shake for 5 seconds
Read absorbance at 405 nm and then at 450 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. Also, the pure water wash step can be omitted when using an automatic washer or automatic plate processor.	