

**Zinc Transporter 8 (ZnT8)
Autoantibody ELISA Kit - Instructions
for use****RSR Limited**

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INTENDED USE

The RSR 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 (type 1 DM). The antigens recognised by these antibodies include insulin, glutamic acid decarboxylase (GAD₆₅ 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

J. M. Wenzlau et al

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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."
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"Bridging-type enzyme-linked immunoassay for zinc transporter 8 autoantibody measurements in adult patients with diabetes mellitus."
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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 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 16-20 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 15 – 2000 u/mL (arbitrary RSR units).

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. Citrate and heparin plasma may be used in the assay. Studies in which EDTA plasma samples were spiked with ZnT8 Ab positive sera showed that lower signals were observed compared with spiked serum from the same donor. In particular OD₄₅₀ values with spiked EDTA plasma were 33% - 65% of spiked serum or 37% - 64% in terms of u/mL (19 samples with spiked serum concentrations ranging from 11 u/mL – 326 u/mL). 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 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 |
|---|------------------------------|
|  | EC Declaration of Conformity |
|  | In Vitro Diagnostic Device |
|  | Catalogue Number |
|  | Lot Number |
|  | Consult Instructions |
|  | Manufactured By |
|  | Sufficient for |
|  | Expiry Date |
|  | Store |
|  | Negative 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 3 months. |
| 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 |
| I | Peroxidase Substrate (TMB) 15 mL Ready for use |
| J | Concentrated Wash Solution 125 mL Concentrate |
| | Dilute 10 X with pure water before use. For example, 100 mL (J) + 900 mL pure water. Store at 2–8°C for up to kit expiry date. |
| K | Stop Solution 12 mL Ready for use |

ASSAY PROCEDURE

Allow all reagents to reach room temperature (20–25°C) on day of use, except ZnT8-Biotin and reconstitution buffer for ZnT8-Biotin. A repeating Eppendorf type pipette is recommended for steps 4, 7, 10 and 11.

| | | |
|--------------|-----------|--|
| Day 1 | 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, shake for approximately 5 seconds on a plate shaker and incubate overnight, for 16-20 hours, at 2–8°C without shaking. |
| Day 2 | 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 cold 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 2–8°C for 1 hour without shaking. |

| | | |
|-----------------|--|--|
| Day 2 continued | 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 (I) 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 30 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 (I) and 100 µL stop solution (K) only. | |

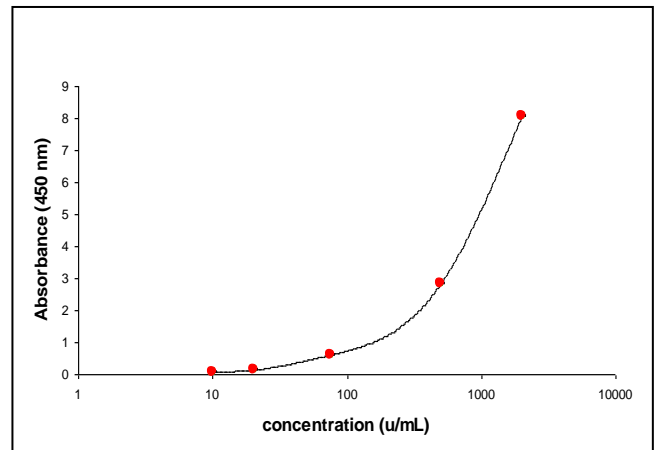
RESULT ANALYSIS

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 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 | 0.068 | 10 | 0.023 | 10 |
| B2 | 0.138 | 20 | 0.043 | 20 |
| B3 | 0.610 | 75 | 0.184 | 75 |
| B4 | 2.838 | 500 | 0.853 | 500 |
| B5 | 8.089 | 2000 | 2.379 | 2000 |
| Negative Control (D) | 0.015 | 0 | 0.008 | 0 |
| Positive Control (CI) | 0.402 | 46 | 0.121 | 46 |
| Positive Control (CII) | 1.221 | 200 | 0.367 | 196 |

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 | < 15 u/mL |
| Positive | ≥ 15 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

In the IASP 2016 study the RSR ZnT8 Ab ELISA kit achieved 99% (n=90) specificity and 72% (n=50) sensitivity.

Assay of 297 healthy blood donor sera gave a mean value of 1.9 ± 3.84 u/mL. 3 sera (1%) were above the assay cut off giving values of 45, 41 and 19 u/mL.

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 1.2 u/mL when the negative control was assigned a value of 1 u/mL.

Inter Assay Precision

| Sample | Mean u/mL (n=20) | CV (%) |
|--------|------------------|--------|
| A | 102 | 9.3 |
| B | 64 | 7.5 |
| C | 26.6 | 8.7 |

Intra Assay Precision

| Sample | Mean u/mL (n=25) | CV (%) |
|--------|------------------|--------|
| 1 | 160 | 6.2 |
| 2 | 63 | 6.2 |
| 3 | 24.5 | 3.5 |

Clinical Accuracy

Sera containing rheumatoid factor (n=26) and sera containing autoantibodies to thyroglobulin (n=20), to thyroid peroxidase (n=24), to aquaporin-4 (n=3) and to the acetylcholine receptor (n=9) were negative for ZnT8 Ab. 4% (n=24) of sera positive for antibodies to the TSH receptor, and 9% (n=23) of sera positive for 21-hydroxylase Ab were positive for ZnT8 Ab using the RSR ZnT8 Ab ELISA kit.

Interference

No interference was observed when samples were spiked with the following materials; haemoglobin at 500 mg/dL, 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 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

| | | |
|---|---|---|
| Day 1 | Allow all reagents and samples to reach room temperature (20-25°C) on day of use, except ZnT8- Biotin and reconstitution buffer for ZnT8-Biotin | |
| | Pipette: | 25 µL Calibrators, controls and patient sera into wells (except blanks)) and shake for 5 seconds |
| | Incubate: | Overnight (16–20 hours) at 2-8°C without shaking |
| Day 2 | Aspirate/Decant: | Plate |
| | Wash: | Plate three times and tap dry on absorbent material ¹ |
| | Pipette: | 100 µL Cold ZnT8-Biotin (reconstituted) into each well (except blanks) |
| | Incubate: | 1 hour at 2-8°C without shaking |
| | 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 30 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. | | |