

Zinc Transporter 8 Autoantibody ELISA

IVD REF **EIA-5764** $\langle \Sigma \rangle$ 96

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DRG Instruments GmbH, Germany Frauenbergstraße. 18, D-35039 Marburg Phone: +49 (0)6421-1700 0, Fax: +49 (0)6421-1700 50 Website: www.drg-diagnostics.de E-mail: drg@drg-diagnostics.de



DRG International, Inc., USA 841 Mountain Ave., Springfield, NJ 07081 Phone: (973) 564-7555, Fax: (973) 564-7556 Website: www.drg-international.com E-mail: corp@drg-international.com Please use only the valid version of the Instructions for Use provided with the kit. Verwenden Sie nur die jeweils gültige, im Testkit enthaltene, Gebrauchsanweisung. Si prega di usare la versione valida delle istruzioni per l'uso a disposizione con il kit. Por favor, se usa solo la version valida de la metodico técnico incluido aqui en el kit. Utilisez seulement la version valide des Instructions d'utilisation fournies avec le kit.

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1 INTENDED USE

The Zinc Transporter 8 Autoantibody ELISA kit is intended for use by professional persons only, for the quantitative determination of Zinc Transporter 8 Autoantibody (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 Autoantibodies 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.

ZnT8 Ab ELISA (EIA-5764) is capable of detecting, and quantifying, autoantibodies specific to R 325 or to W 325, or to residue 325 non-specific variants.

2 REFERENCES / LITERATURE

- J. M. Wenzlau et al "The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type I diabetes." PNAS 2007 104:17040-17045
- P. Achenbach et al "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.

3 ASSAY PRINCIPLE

In the Zinc Transporter 8 Autoantibody 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 405 nm may be used.

The measuring interval is 15 – 2000 U/mL (arbitrary DRG units).

4 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 μ L is sufficient for one assay (duplicate 25 μ L 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 OD_{450} 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.

5 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 450 nm and 405 nm.
- ELISA Plate shaker, capable of 500 shakes/min (not an orbital shaker).
- ELISA Plate cover.

6 PREPARATION OF REAGENTS SUPPLIED

Store unopened kits and all components at 2 °C - 8 °C.

	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 °C -25 °C) for at least 30 minutes before opening.
A	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 °C - 8 °C for up to 3 months.
	Calibrators
B1-5	10, 20, 75, 500, 2000 U/mL (units are arbitrary DRG units)
	5 x 0.7 mL
	Ready for use
	Positive Controls I & II
C1-2	(see label for concentration range)
	2 x 0.7 mL
	Ready for use
	Negative Control
D	0.7 mL
	Ready for use
	ZnT8-Biotin
	3 vials
E	
	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 °C - 8 °C for up 3 days after reconstitution.
_	Reconstitution Buffer for Zn18-Biotin
F	2 x 15 mL, coloured red
	Ready for use
^	Streptavidin Peroxidase (SA-POD)
9	
	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 °C - 8 °C for up to 16 weeks after dilution.
	Diluent for SA-POD
H	15 mL
	Ready for use
	Peroxidase Substrate (TMB)
1	15 mL
	Ready for use
	Concentrated Wash Solution
	125 mL Concentrate
J	Dilute 10 X with pure water before use.
	For example, 100 mL (J) + 900 mL pure water.
	Store at 2 °C - 8 °C for up to kit expiry date.
	Stop Solution
K	12 mL
	Ready for use

7 ASSAY PROCEDURE

Allow all reagents to reach room temperature (20 °C - 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 μ L of calibrators (B1-5), controls (C1-2 and D) and patients' sera into respective wells, in duplicate is recommended, 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 $^{\circ}$ C - 8 $^{\circ}$ 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 the 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 °C - 8 °C for 1 hour without shaking.
	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 (20 $^{\circ}$ C - 25 $^{\circ}$ 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 (I) into each well (including blanks) and incubate in the dark at room temperature (20 °C - 25 °C) 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 405 nm, 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 .

8 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 DRG 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 μ L of sample plus 135 μ L 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.

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

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

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



9 ASSAY CUT OFF

Cut off	U/mL		
Negative	< 15 U/mL		
Positive	≥ 15 U/mL		

This cut off has been validated at DRG. 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.

10 CLINICAL EVALUATION

10.1 Clinical Specificity and Sensitivity

In the IASP 2016 study the 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.

10.2 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.

10.3 Inter Assay Precision

Sample	Mean U/mL (n=20)	CV (%)	
А	102	9.3	
В	64	7.5	
С	26.6	8.7	

10.4 Intra Assay Precision

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

10.5 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 ZnT8 Ab ELISA (EIA-5764) kit.

10.6 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.

11 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

Diluent for SA-POD

Hazard statement(s)

EUH208: Contains 2-Chloroacetamide. May produce an allergic reaction. 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 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.

12 ASSAY PLAN

	Allow all reagents and samples to reach room temperature (20 °C - 25 °C) on day of use, except ZnT8-Biotin and reconstitution buffer for ZnT8-Biotin				
Day 1	Pipette:	$25\ \mu L$ Calibrators, controls and patient sera into wells (except blanks)) and shake for 5 seconds			
	Incubate:	Overnight (16 - 20 hours) at 2 °C - 8 °C without shaking			
	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 °C - 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)				
Jay 2	Incubate:	20 minutes at room temperature (20 $^\circ\text{C}$ - 25 $^\circ\text{C})$ on an ELISA plate shaker at 500 shakes/min			
	Aspirate/Decant:	Plate			
Wash: Plate three times, rinse with pure water and tap dry on absorb		Plate three times, rinse with pure water and tap dry on absorbent material ¹			
Pipette: 100 µL TMB into each well (including blanks)		100 μL TMB into each well (including blanks)			
	Incubate: 20 minutes at room temperature (20 °C - 25 °C) in the dark				
	Pipette: 100 µL Stop Solution into each well (including blanks) and shake for 5 set				
	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.				

Symbol	English	Deutsch	Italiano	Español	Français	Português
((European Conformity	CE-Konformitäts- kennzeichnung	Conformità europea	Conformidad europea	Conformité normes européennes	Conformidade Europeia
[]i]	Consult instructions for use *	Gebrauchsanweisung beachten *	Consultare le istruzioni per l'uso	Consulte las instrucciones de uso	Consulter les instructions d'utilisation	Consultar as instruções de uso
IVD	In vitro diagnostic medical device *	In-vitro-Diagnostikum *	Diagnostica in vitro	Diagnóstico in vitro	Diagnostic in vitro	Dispositivo médio para diagnóstico vitro
REF	Catalogue number *	Katalognummer *	No. di Cat.	No de catálogo	Référence	Número de catálo
LOT	Batch code *	Chargen-bezeichnung *	Lotto no	Número de lote	No. de lot	Código do lote
Σ	Contains sufficient for <n> tests *</n>	Ausreichend für <n> Prüfungen *</n>	Contenuto sufficiente per "n" saggi	Contenido suficiente para <n> ensayos</n>	Contenu suffisant pour "n" tests	Suficiente para < determinações
X	Temperature limit *	Temperaturgrenzwerte *	Temperatura di conservazione	Temperatura de conservacion	Temperature de conservation	Limites de temperatura
\sum	Use-by date *	Verwendbar bis *	Data di scadenza	Fecha de caducidad	Date limite d'utilisation	Prazo de validad
^	Manufacturer *	Hersteller *	Fabbricante	Fabricante	Fabricant	Fabricante
	Distributor *	Vertriebspartner *	Distributore	Distribuidor	Distributeur	Distribuidor
\sim	Date of manufacture *	Herstellungsdatum *	Data di produzione	Fecha de fabricación	Date de production	Data de fabricaç
\$	Biological risks *	Biologische Risiken *	Rischi biologici	Riesgos biológicos	Risques biologiques	Riscos biológico
\triangle	Caution *	Achtung *	Attenzione	Precaución	Attention	Cuidado
UDI	Unique device Identifier *	eindeutige Produktidentifizierung *	Identificativo unico del dispositivo*	Identificación exclusiva del dispositivo *	Identifiant de dispositif unique*	Identificador único do dispositivo *
RUO	For research use only	Nur für Forschungszwecke	Solo a scopo di ricerca	Sólo para uso en investigación	Seulement dans le cadre de recherches	
Content	Content	Inhalt	Contenuto	Contenido	Conditionnement	Conteúdo
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantità	Volumen/Número	Volume/Quantité	Volume / Quantidade