DIAGNOSTIKA GMBH

Instructions for Use

Zinc Transporter 8 (ZnT8) Antibody ELISA

Enzyme Immuno Assay for the Quantitative Determination of ZnT8 Autoantibodies in Serum

CE

IVD

REF EA102/96

12 x 8

2 – 8 °C

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Contents

Symbols

I V D	In-Vitro-Diagnostic Device	CE	EC Declaration of Conformity
CONT	Contents		Expiry Date
LOT	Lot Number	+2 +8 °C	Store
	Manufactured by	Σ	Sufficient for
REF	Catalogue Number		Consult Instructions

Hazard Pictograms

Warning

Danger

1. Introduction and Principle of the Test

The ZnT8 autoantibody (ZnT8 Ab) ELISA kit is intended for use for the quantitative determination of ZnT8 autoantibodies 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 autoantibodies 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 autoantibody ELISA is capable of detecting, and quantifying, autoantibodies specific to R 325 or to W 325, or to residue 325 non-specific variants.

In ZnT8 Ab ELISA, ZnT8 autoantibodies 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 autoantibodies bound to the ZnT8 coated wells. ZnT8 Biotin is added in a 2nd incubation step where, through the ability of ZnT8 autoantibodies 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 3,3',5,5' – tetramethylbenzidine (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 autoantibody in the test sample. Reading at 405 nm allows quantitation of high absorbances. Low values (less than 50 units per ml) should be read off the 450 nm calibration curve. The measuring range is 10 – 2000 u/ml.

The following patents apply: European patents EP 1563071 B1 and EP 2118309 B1, US patents 7,851,164 B2 and 9,023,984 B2, Chinese patents CN1738900B and ZL 200780051859.3, India patent 279741 and Japanese patents 4498144 and 5694668.

2. Precautions

- For in vitro use only.
- Some reagents contain sodium azide as preservative (<0.1%). Avoid skin contact.
- All reagents of human origin used in this kit are tested for HIV I/II antibodies, HCV and HBsAg and found to be negative. However, because no test method can offer complete assurance that infectious agents are absent, these reagents should be handled as potentially biohazardous materials.
- Material of animal origin used in the preparation of the kit have been obtained from certified healthy animals but these materials should be handled as potentially infectious.

3. Storage and Stability

On arrival, store the kit at 2-8 °C. Once opened the kit is stable until its expiry date. For stability of prepared reagents refer to Preparation of Reagents.

4. Contents of the Kit

4.1 **ZnT8 Coated Wells** **STRIPS** 12 strips
8 wells per strip
coated with ZnT8

4.2 **Calibrators A – E** **CAL A** – **CAL E** 5 vials
0.7 ml each, ready for use

Concentrations (arbitrary Units):

Calibrator	A	B	C	D	E
U/ml	10	20	75	500	2,000

4.3 **Positive Controls** **CONTROL I** **CONTROL II** 2 vials
0.7 ml, ready for use
for concentration range see included QC-Certificate sheet

4.4 **Negative Control** **CON -** 1 vial
0.7 ml, ready for use

4.5 **ZnT8-Biotin** **ZnT8-BIOTIN** 3 vials
5.5 ml per vial, lyoph.

4.6 **Reconstitution Buffer** **RECONST** 2 vials
15 ml, coloured red, ready for use
for reconstituting ZnT8-Biotin

4.7 **Streptavidin-peroxidase (SA-POD)** **SA-POD**  Warning 1 vial
0.7 ml, 20 x concentrated

4.8 **Diluent** **DIL** 1 vial
15 ml, ready for use
for diluting SA-POD

4.9 **Substrate** **SUB**  Danger 1 vial
15 ml, tetramethyl benzidine (TMB), ready for use

4.10 **Wash Buffer** **WASH** 1 vial
125 ml, 10 x concentrated

4.11 **Stop Solution** **STOP** 1 vial
12 ml, ready for use

Additional materials and equipment required but not provided:

- Pipettes for 25 µl and 100 µl
- ELISA plate cover
- ELISA plate shaker capable of 500 shakes per min (not an orbital shaker)
- Pure water
- Microtiter plate reader (450 nm and 405 nm)

5. Specimen Collection and Storage

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). Subsequent freezing and thawing or increase in storage temperature must be avoided. Incorrect storage of serum samples can lead to loss of ZnT8 autoantibodies.
Citrate and heparin plasma may be used in the assay. Studies with EDTA plasma indicate lower values as compared to serum samples.
Do not use grossly haemolysed or lipaemic serum samples.
When required, thaw test sera at room temperature and mix gently to ensure homogeneity. Centrifuge the serum prior to assay (preferably for 5 min at 10-15,000 g in a microfuge) to remove any particulate matter. Please do not omit this centrifugation step if sera are cloudy or contain particulates.

6. Limitations

- Lipaemic or grossly haemolysed sera should not be used.
- Always centrifuge cloudy sera immediately before assay.
- Sera with unusually low or unusually high protein concentrations give erroneous results.
- Always store frozen serum samples carefully and do not allow increases in temperature above -20 °C. Incorrect storage can lead to loss of antibody activity.

7. Test Procedure

7.1. Preparation of Reagents

MT strips **STRIPS**

Before opening the packet of strip wells, allow it to stand at room temperature for at least 30 minutes. After opening, keep any unused wells in the original foil packet (reseal with adhesive tape) and in the self-seal plastic bag with the desiccant provided. Store at 2-8 °C for up to 3 months.

ZnT8-Biotin **ZnT8-BIOTIN**

Immediately before use reconstitute the contents of each vial with 5.5 ml cold ZnT8 Reconstitution Buffer (coloured red). If more than 1 vial of ZnT8-Biotin is going to be used, pool the contents of each vial after reconstitution and mix gently before use. Store at 2-8 °C for up to 3 days after reconstitution.

Streptavidin-Peroxidase (SA-POD) **SA-POD**

Dilute the concentrate 1 in 20 with the Diluent provided (e.g. 0.5 ml SA-POD + 9.5 ml Diluent). Store at 2-8 °C for up to 16 weeks after dilution.

Wash Buffer **WASH**

Dilute 1 in 10 with pure water before use (e.g. 100 ml concentrate + 900 ml pure water). Store at 2-8 °C after dilution up to kit expiry date.

7.2. Assay Procedure

Calculate the number of individual ELISA plate wells needed for the assay. Allow all the reagents supplied, including the appropriate number of packets of strips, to reach room temperature (at least 30 min), **except ZnT8 Biotin and ZnT8 Biotin Reconstitution Buffer!** Remove the number of strip wells required and fit them firmly into the frame provided. Negative and Positive Controls in duplicate must always be included in each assay run.

1. Pipette each 25 µl (in duplicate) of Calibrators A - E, Negative and Positive Controls and test sera into the appropriate wells. Leave one well empty for blank.

2. Cover the plate, shake for approximately 5 seconds on a plate shaker and incubate overnight, for 16-20 hours at 2-8°C without shaking.

3. After the overnight incubation, aspirate or discard the reagent from the wells, and wash the wells three times with diluted Wash Buffer. Tap the inverted wells gently on a clean dry absorbent surface to remove any droplets of Wash Buffer.

4. Pipette 100 µl of **cold reconstituted** ZnT8-Biotin into each well (except blank), cover the plate and incubate for 1 hour at 2-8 °C without shaking.

5. Repeat wash step 3.

6. Pipette 100 µl of diluted SA-POD into each well (except blank). Cover the plate and incubate for 20 minutes at room temperature with shaking on an ELISA plate shaker (500 shakes per min).

7. Repeat wash step 3. Use one additional wash step with pure water to remove any foam before finally tapping the inverted wells dry.

8. Pipette 100 µl of Substrate (TMB) into each well (including blank) and incubate for 20 minutes at room temperature in the dark without shaking.

9. Stop the substrate reaction by addition of 100 µl of Stop Solution to each well (including blank) and shake the plate for about 5 seconds on a plate shaker to ensure uniformity of the solution in each well.

10. Read within 30minutes the absorbance at 405 nm and then at 450 nm using an ELISA plate reader blanked against the well containing 100 µl Substrate plus 100 µl of Stop Solution only.

8. Calculation of Results

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 autoantibody concentrations in patient sera can then be read off the calibration curve.

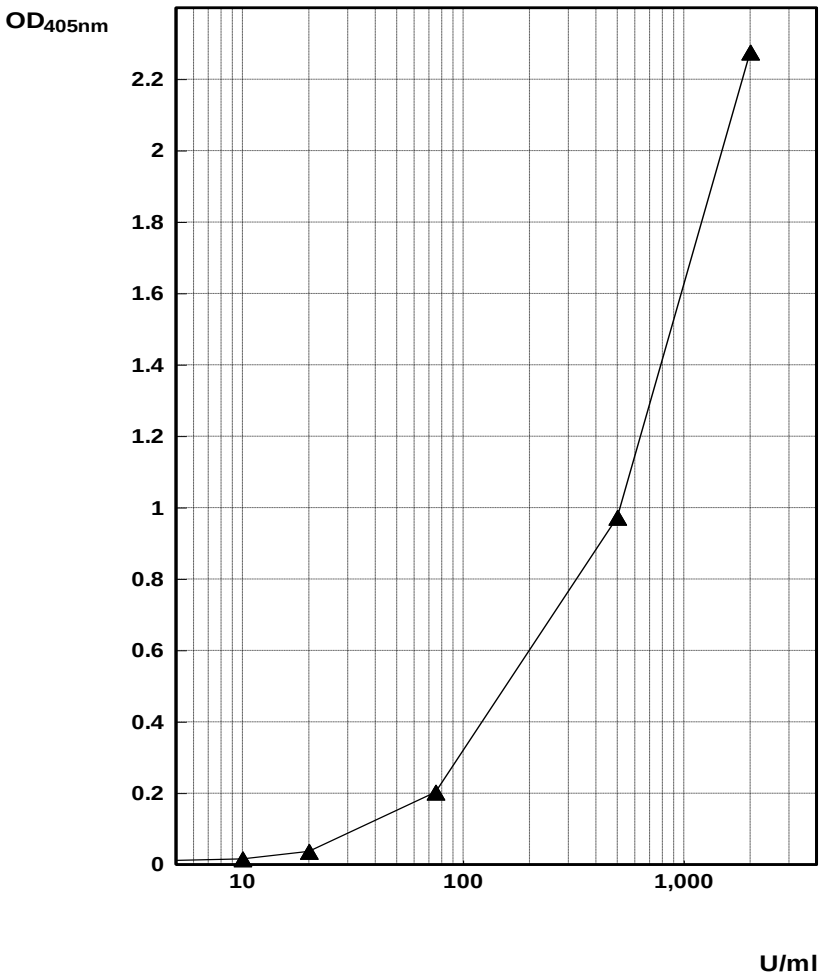
Cubic spline, 4 parameter or similar iteration procedures are recommended for evaluation of the standard curve

Other data reduction systems can be used. The negative control 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. 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 Example

Typical results are shown in the following table.

	OD 405 nm	OD 450 nm	Conc. U/ml
Negative Control	0.002	0.008	0
Calibrator A	0.016	0.060	10
Calibrator B	0.037	0.134	20
Calibrator C	0.203	0.673	75
Calibrator D	0.973	3.222	500
Calibrator E	2.276	7.738	2,000
Positive Control I	0.137	0.463	60
Positive Control II	0.477	1.596	134



9. Clinical Evaluation

In the IASP 2015 study the ZnT8 Ab ELISA kit achieved 97% (n=90) specificity and 76% (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. Expected Values

Negative	< 15 U/ml
Positive	≥ 15 U/ml

This cut off has been validated. 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.

11. 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

Pipetting Scheme

Allow all the reagents supplied, including the appropriate number of packets of strips, to reach room temperature (at least 30 min),
except ZnT8-Biotin and Reconstitution Buffer!

		B_0	Calibrator A - E	Positive Control I/II	Patient Sample
Negative Control	µl	25			
Calibrators	µl		25		
Positive Controls	µl			25	
Patient Serum	µl				25

Incubate overnight (16 – 18 hours) at 2-8 °C without shaking

Aspirate / discard and wash three times with each 300 µl Wash Buffer

Cold ZnT8 Biotin	µl	100	100	100	100

Incubate for 1 hour at 2-8 °C without shaking

Aspirate / discard and wash three times with each 300 µl Wash Buffer

Diluted SA-POD	µl	100	100	100	100

Incubate for 20 min at room temperature on a shaker 500 shakes/min

Aspirate / discard and wash three times with each 300 µl Wash Buffer
Wash once with 300 µl pure water

TMB-Substrate	µl	100	100	100	100

Incubate for 20 minutes at room temperature in the dark without shaking

Stop Solution	µl	100	100	100	100

5 sec shaking on an ELISA plate shaker

Reading of absorbance at 405 nm / 450 nm within 30 minutes