Arbeitsanleitung / Manual

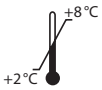
IDK® Zonulin ELISA Kit

Zur in-vitro-Bestimmung von Zonulin in Stuhl

For the in vitro determination of zonulin in stool

Gültig ab / Valid from 2015-09-24

REF **K 5600**



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1. INTENDED USE

This ELISA is intended for the quantitative determination of zonulin in stool. For Research Use only and not for diagnostic procedures.

2. INTRODUCTION

Zonulin is a novel human protein analogue to the zonula occludens toxin derived from *Vibrio cholerae* which participates in tight junctions between cells of the wall of the digestive tract. Zonulin binds to a specific receptor on the surface of intestinal epithelia and triggers a cascade of biochemical events which induces tight junction disassembly and a subsequent permeability increase of the intestinal epithelia, allowing some substances to pass through and activate immune reactions.

Fasano and his co-workers found that the zonulin-zonulin-receptor-system is more activated in celiac disease and type 1 diabetes mellitus patients. Patients with active celiac disease showed higher levels of zonulin and anti-zonulin antibodies compared to non-celiac patients and patients in remission, who were on a gluten-free diet.

Concerning the autoimmune type 1 diabetes, in experiments with rats it could be demonstrated that elevated zonulin levels as well as increased intestinal permeability precede a type 1 diabetes disease. Conversely, type 1 diabetes could be prevented by inhibition of zonulin in animal experiments.

In addition, it was reported that many people who suffer from celiac disease also suffer from other autoimmune disorders. It is suggested that increased levels of zonulin are a contributing factor to the development of celiac disease and other autoimmune disorders such as insulin dependent diabetes, multiple sclerosis and rheumatoid arthritis.

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity
K 5600	MTP	Microtiter plate, coated	12 x 8 wells
K 5600	WASHBUF	Wash buffer concentrate, 10 x	2 x 100 ml
K 5600	DIL	Dilution buffer concentrate, 2.5 x	2 x 100 ml
K 5600	TRACER	Tracer, biotinylated zonulin, concentrate, 100 x	1 x 300 µl
K 5600	CONJ	Conjugate, peroxidase-labeled streptavidin, concentrate, 100 x	1 x 200 µl

Cat. No.	Label	Kit components	Quantity
K 5600	STD	Standards (lyophilized)	4 x 5 vials
K 5600	CTRL1	Control (lyophilized)	4 x 1 vial
K 5600	CTRL2	Control (lyophilized)	4 x 1 vial
K 5600	SUB	TMB substrate (Tetramethylbenzidine), ready to use	1 x 15 ml
K 5600	STOP	ELISA stop solution, ready to use	1 x 15 ml

For reorders of single components, use the catalogue number followed by the label as product number.

4. MATERIAL REQUIRED BUT NOT SUPPLIED

- Ultra pure water*
- Calibrated precision pipettors and 10–1000 µl tips
- Foil to cover the microtiter plate
- Horizontal microtiter plate shaker (available via Immundiagnostik upon request)
- Multi-channel pipets or repeater pipets
- Centrifuge, 3000 *g*
- Vortex
- Standard laboratory glass or plastic vials, cups, etc. made from polypropylene
- Microtiter plate reader

* Immundiagnostik AG recommends the use of Ultra Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25 °C (≥ 18.2 MΩ cm).

5. PREPARATION AND STORAGE OF REAGENTS

- To run the assay more than once, ensure that reagents are stored at the conditions stated on the label. **Prepare only the appropriate amount necessary for each run.** The kit can be used up to 4 times within the expiry date stated on the label.
- Reagents with a volume less than **100 µl** should be centrifuged before use to avoid loss of volume.
- **Preparation of the wash buffer:** The **wash buffer concentrate (WASHBUF)** should be diluted with ultra pure water **1:10** before use (100 ml WASHBUF + 900 ml ultra pure water), mix well. Crystals could occur due to high salt concentration in the stock solutions. The crystals must be redissolved at room

temperature or in a water bath at 37 °C before dilution of the buffer solutions. The **WASHBUF** is stable at **2–8 °C** until the expiry date stated on the label. **Wash buffer** (1:10 diluted WASHBUF) can be stored in a closed flask at **2–8 °C for one month**.

- **Preparation of the dilution buffer:** The **DIL** (dilution buffer concentrate) should be diluted with **ultra pure water 1:2.5** before use (100 ml DIL + 150 ml ultra pure water), mix well. Crystals can occur due to high salt concentration in the stock solutions. The crystals must be redissolved at 37 °C in a water bath before dilution. The **DIL** is stable at **2–8 °C** until the expiry date stated on the label. Dilution buffer (1:2.5 diluted DIL) can be stored in a closed flask at **2–8 °C** for **one month**.
- **Preparation of standards and controls:** The **lyophilized STD** (standards) and **CTRL** (controls) are stable at **2–8 °C** until the expiry date stated on the label. Standards and controls have to be **reconstituted with ultra pure water** (volume and concentration, see product specification). Allow the vial content to dissolve for 10 minutes and then mix thoroughly to ensure complete reconstitution. **Reconstituted standards and controls are not stable.**
- **Preparation of the tracer:** The **tracer concentrate (TRACER)** must be diluted **1:101 in dilution buffer** (150 µl TRACER + 15 ml dilution buffer) immediately before use. The TRACER is stable at **2–8 °C** until expiry date stated on the label. **Tracer** (1:101 diluted TRACER) **is not stable and cannot be stored.**
- **Preparation of the conjugate:** The **conjugate concentrate (CONJ)** must be diluted **1:101** in dilution buffer (100 µl CONJ + 10 ml dilution buffer). The CONJ is stable at **2–8 °C** until expiry date stated on the label. **Conjugate** (1:101 diluted CONJ) **is not stable and cannot be stored.**
- All other test reagents are ready to use. Test reagents are stable until the expiry date (see label of test package) when stored at **2–8 °C**.

6. SAMPLECOLLECTION AND PREPARATION

The sample stability is as follows:

Raw stool

4 days at room temperature (15–30 °C) as well as three months at -20 °C.

Stool extracts (1:50)

7 days at -20 °C.

Extraction of the stool samples

Dilution buffer is used as a sample extraction buffer. We recommend the following sample preparation:

Stool Sample Application System (SAS) (Cat. No.: K 6998SAS)

Stool sample tube – Instructions for use

Please note that the dilution factor of the final stool suspension depends on the amount of stool sample used and the volume of the buffer.

SAS with 0.75 ml extraction buffer:

Applied amount of stool:	15 mg
Buffer Volume:	0.75 ml
Dilution Factor:	1:50

Please follow the instructions for the preparation of stool samples using the SAS as follows:

a) The raw stool sample has to be thawed. For particularly heterogeneous samples we recommend a mechanical homogenisation using an applicator, inoculation loop or similar device.

b) Fill the **empty sample tube** with **0.75 ml** of ready to use extraction buffer before using it with the sample. Important: Allow the extraction buffer to reach room temperature.

c) Unscrew the tube (yellow part of cap) to open. Insert the orange dipstick into the sample. The lower part of the dipstick has notches which need to be covered completely with stool after inserting it into the sample. Place dipstick back into the tube. When putting the stick back into the tube, excess material will be stripped off, leaving 15 mg of sample to be diluted. Screw tightly to close the tube.

d) Shake the tube well until no stool sample remains in the notches. Important: Please make sure that you have a maximally homogenous suspension after shaking. Especially with more solid samples, soaking the sample in the tube with buffer for ~ 10 minutes improves the result.

e) Allow sample to stand for ~10 minutes until sediment has settled. Floating material like shells of grains can be neglected.

f) Carefully unscrew the complete cap of the tube including the blue ring plus the dipstick. Discard cap and dipstick. Make sure that the sediment will not be dispersed again.

Dilution Factor: **1:50**

Sample preparation

Pipet **150 µl of each stool sample supernatant** and **150 µl of tracer** in labeled reaction tubes and mix well.

Preparation of standards and controls

Transfer **150 µl** of **STD** or **CTRL** in the correspondingly labeled reaction tubes, add **150 µl** of **tracer** and mix well.

7. ASSAY PROCEDURE

Principle of the test

This assay is based on the method of competitive ELISA. As a first preparation step, a biotinylated zonulin tracer is added to the samples, standards and controls. Afterwards, aliquots of the treated samples, standards and controls are transferred and incubated in microtiter plate wells coated with polyclonal anti-zonulin antibodies. During the incubation, the free target antigen in the samples competes with the biotinylated zonulin tracer for the binding of the polyclonal anti-zonulin antibodies immobilized on the microtiter plate wells. The unbound components are removed by a washing step. During a second incubation step, peroxidase-labeled streptavidin, which binds to the biotinylated zonulin tracer, is added into each microtiter well. After a washing step to remove the unbound components, the peroxidase substrate tetramethylbenzidine is added. Finally, the enzymatic reaction is terminated by an acidic stop solution. The colour changes from blue to yellow and the absorbance is measured in the photometer at 450 nm. The intensity of the yellow colour is inverse proportional to the zonulin concentration in the sample; this means, high zonulin concentration in the sample reduces the concentration of the biotinylated zonulin tracer bound to the immobilized anti-zonulin antibodies and lowers the photometric signal. A dose response curve of absorbance unit (optical density, OD at 450 nm) vs. concentration is generated using the values obtained from the standard.

Test procedure

Prior to use, allow all reagents and samples to come to room temperature (15–30 °C) and mix well.

Take as many microtiter strips (PLATE) as needed from kit. Store unused strips in the closed aluminium packaging at 2–8 °C. Strips are stable until the expiry date stated on the label.

For automated ELISA processors, the given protocol may need to be adjusted according to the specific features of the respective automated platform. For further details please contact your supplier or Immundiagnostik AG.

We recommend to carry out the tests in duplicate.

1.	Add **100 µl** of the prepared **standards, controls or samples** into each well.
2.	Cover the strips and incubate for **1 hour shaking** on a horizontal shaker at 350 rpm with an orbit of 2 mm at room temperature (15–30 °C).
3.	Decant the contents of each well. Wash the microtiter plate **5 x** with **250 µl wash buffer**. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper to remove excess solution.
4.	Add **100 µl** of **conjugate** into each well.
5.	Cover the strips and incubate for **1 hour shaking** on a horizontal shaker at 350 rpm with an orbit of 2 mm at room temperature (15–30 °C).
6.	Decant the contents of each well. Wash the microtiter plate **5 x** with **250 µl wash buffer.** After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper to remove excess solution.
7.	Add **100 µl** of **SUB** (substrate solution) into each well.
8.	Incubate for **10–20 minutes** at room temperature (15–30 °C)*.
9.	Add **100 µl** of **STOP** (stop solution) into each well and mix shortly in the ELISA reader using the shake option.
10.	Determine **absorption immediately** with an ELISA reader at **450 nm** against 620 nm (or 690 nm) as a reference. If no reference wavelength is available, read only at 450 nm. If the extinction of a sample or standard exceeds the range of the photometer, absorption must be measured immediately at **405 nm** against 620 nm as a reference.

* The intensity of the colour change is temperature sensitive. We recommend observing the colour change and stopping the reaction upon good differentiation.

8. RESULTS

The following algorithms can be used alternatively to calculate the results. We recommend using the "4 parameter algorithm".

1. **4 parameter algorithm**

   It is recommended to use a linear ordinate for the optical density and a logarithmic abscissa for the concentration. When using a logarithmic abscissa, the zero standard must be specified with a value less than 1 (e. g. 0.001).

2. **Point-to-point calculation**

   We recommend a linear ordinate for the optical density and a linear abscissa for the concentration.

3. **Spline algorithm**

   We recommend a linear ordinate for the optical density and a linear abscissa for the concentration.

The plausibility of the duplicate values should be examined before the automatic evaluation of the results. If this option is not available with the programme used, the duplicate values should be evaluated manually.

Stool samples

The obtained zonulin levels of stool samples have to be multplied with the dilution factor of **50**.

In case **another dilution factor** has been used, multiply the obtained result with the dilution factor used.

9. LIMITATIONS

Samples with concentrations above the measurement range (see definition below) must be further diluted and re-assayed. Please consider this greater dilution when calculating the results.

Samples with concentrations lower than the measurement range (see definition below) cannot be clearly quantified.

The upper limit of the measurement range can be calculated as:

highest concentration of the standard curve × *sample dilution factor to be used*

The lower limit of the measurement range can be calculated as:

LoB × *sample dilution factor to be used*

10. QUALITY CONTROL

Immundiagnostik recommends the use of external controls for internal quality control, if possible.

Control samples should be analysed with each run. Results, generated from the analysis of control samples, should be evaluated for acceptability using appropriate statistical methods. The results for the patient samples may not be valid if within the same assay one or more values of the quality control sample are outside the acceptable limits.

Reference range

Based on Immundiagnostik studies of stool samples of apparently healthy persons (n = 40), a median value of 61 ng/ml (± 46 ng/ml) was estimated.

We recommend each laboratory to establish its own reference range.

11. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

Intra-Assay (n = 24)

Sample	Zonulin [ng/ml]	VK [%]
1	117.7	6.2
2	130.9	5.9
3	38.3	3.2

Inter-Assay (n = 16)

Sample	Zonulin [ng/ml]	VK [%]
1	36.4	12.7
2	162.9	13.9

Dilution recovery

Two stool samples were diluted and analyzed. The results are shown below (n = 2):

Sample	Dilution	Zonulin expected [ng/ml]	Zonulin measured [ng/ml]
A	1:50		58.5
	1:100	29.3	20.3
	1:200	14.6	10.6
	1:400	7.3	6.7
B	1:50		169
	1:100	84.5	80
	1:200	42.3	42.5
	1:400	21.1	24.1

Analytical Sensitivity

Limit of blank, LoB	0.105 ng/ml
Limit of detection, LoD	0.241 ng/ml
Limit of quantitation, LoQ	0.241 ng/ml

The evaluation was performed according to the CLSI guideline EP-17-A2.

12. PRECAUTIONS

- All reagents in the kit package are for *in vitro* diagnostic use only.
- Human materials used in kit components were tested and found to be negative for HIV, Hepatitis B and Hepatitis C. However, for safety reasons, all kit components should be treated as potentially infectious.
- Kit reagents contain sodium azide or Proclin as bactericides. Sodium azide and Proclin are toxic. Substrates for the enzymatic colour reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
- The stop solution consists of diluted sulphuric acid, a strong acid. Although diluted, it still must be handled with care. It can cause burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped up immediately with copious quantities of water. Do not breath vapour and avoid inhalation.

13. TECHNICAL HINTS

- Do not interchange different lot numbers of any kit component within the same assay. Furthermore we recommend not assembling wells of different microtiter plates for analysis.
- Control samples should be analyzed with each run.
- Reagents should not be used beyond the expiration date stated on kit label.
- Substrate solution should remain colourless until use.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Avoid foaming when mixing reagents.
- Do not mix plugs and caps from different reagents.
- The assay should always be performed according the enclosed manual.

14. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and distributed according to the IVD guidelines of 98/79/EC.
- The guidelines for medical laboratories should be followed.
- *IDK*® is a trademark of Immundiagnostik AG.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which is not coordinated with the producer, may influence the results of the test. Immundiagnostik AG can therefore not be held responsible for any damage resulting from incorrect use.
- Warranty claims and complaints regarding deficiencies must be logged within 14 days after receipt of the product. The product should be send to Immundiagnostik AG along with a written complaint.

15. REFERENCES

1. Fasano, A, T Not, W Wang, S Uzzau, I Berti, A Tommasini, and S E Goldblum. 2000. "Zonulin, a Newly Discovered Modulator of Intestinal Permeability, and Its Expression in Coeliac Disease." *Lancet* **355** (9214) (April 29): 1518–9. doi:10.1016/S0140-6736(00)02169-3.

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Used symbols:

	Temperature limitation	REF	Catalogue Number
IVD	In Vitro Diagnostic Medical Device	→REF	To be used with
	Manufacturer		Contains sufficient for <n> tests
LOT	Lot number		Use by