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Manual

IDK® Zonulin ELISA

For the in vitro determination of zonulin family peptides (ZFP) in serum

Valid from 2022-06-22

REF **K 5601** Σ 96

REF **K 5601.20** Σ 20 x 96

+8°C / +2°C

IVD

CE

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1. INTENDED USE

This ELISA is intended for the quantitative determination of zonulin family peptides (ZFP) in serum. For *in vitro* diagnostic use only.

2. INTRODUCTION

Zonulin is a human protein analogue to the zonula occludens toxin derived from *Vibrio cholerae* which regulates tight junctions 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 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.

An increased intestinal permeability, also colloquially called 'leaky gut', is nowadays associated with the metabolic syndrome, obesity, and several autoimmune, inflammatory, and neoplastic diseases. Based on evidence, leaky gut plays a meaningful role in diseases such as multiple sclerosis, rheumatoid arthritis, asthma, and inflammatory bowel diseases.

The polyclonal anti-body used in our ELISA is based on the zonulin sequence as published by Wang (Journal of Cell Science, 2000) and di Pierro (Journal of Biological Chemistry, 2001).

Correspondingly, the readings of ***IDK***® Zonulin ELISA detecting zonulin family peptides correlate well - as already found in many papers - with established metabolic traits linked to increased gut permeability, such as insulin resistance and obesity.

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity for cat. no.	
			K 5601	K 5601.20
K 5601	PLATE	Microtiter plate, pre-coated	12 x 8 wells	20 x 12 x 8 wells
K 0001.C.100	WASHBUF	Wash buffer concentrate, 10x	2 x 100 ml	40 x 100 ml

Cat. No.	Label	Kit components	Quantity for cat. no.	
			K 5601	K 5601.20
K 5601	DIL	Dilution buffer, ready-to-use	1 x 100 ml	20 x 100 ml
K 5601	TRACER	Tracer concentrate, biotinylated ZFP	1 x 300 µl	20 x 300 µl
K 5601	CONJ	Conjugate concentrate, peroxidase-labelled streptavidin	1 x 200 µl	20 x 200 µl
K 5601	STD	Standards, lyophilised (see specification for concentrations)	4 x 5 vials	25 x 5 vials
K 5601	CTRL1	Control, lyophilised (see specification for range)	4 x 1 vial	25 x 1 vial
K 5601	CTRL2	Control, lyophilised (see specification for range)	4 x 1 vial	25 x 1 vial
K 0002.15	SUB	Substrate (tetramethyl-benzidine), ready-to-use	1 x 15 ml	20 x 15 ml
K 0003.15	STOP	Stop solution, ready-to-use	1 x 15 ml	20 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

- Ultrapure water*
- Calibrated precision pipettors and 10–1000 µl single-use tips
- Foil to cover the microtiter plate
- Horizontal microtiter plate shaker
- Multi-channel pipets or repeater pipets
- Vortex
- Standard single-use laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader (required filters see chapter 7)

* Immundiagnostik AG recommends the use of ultrapure 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)** has to be diluted with ultrapure water **1:10** before use (100 ml WASHBUF + 900 ml ultrapure water), mix well. Crystals could occur due to high salt concentration in the concentrate. Before dilution, the crystals have to be redissolved at room temperature or in a water bath at 37 °C. The **WASHBUF** can be used until the expiry date stated on the label when stored at **2–8 °C**. **Wash buffer** (1:10 diluted WASHBUF) can be stored in a closed flask at **2–8 °C for 1 month**.
- The **lyophilised standards (STD)** and **controls (CTRL)** can be used until the expiry date stated on the label when stored at **2–8 °C**. **Reconstitution** details are given in the **specification data sheet**. **Standards and controls** (reconstituted STD and CTRL) **are not stable and cannot be stored.**
- **Preparation of the tracer:** The **tracer concentrate (TRACER)** has to be diluted **1:101 in dilution buffer** (e.g. 150 µl TRACER + 15 ml DIL) immediately before use. The TRACER can be used until the expiry date stated on the label when stored at **2–8 °C**. **Tracer** (1:101 diluted TRACER) **is not stable and cannot be stored.**
- **Preparation of the conjugate:** Immediately before use, the **conjugate concentrate (CONJ)** has to be diluted **1:101** in dilution buffer (e.g. 100 µl CONJ + 10 ml DIL). The **CONJ** can be used until the expiry date stated on the label when stored at **2–8 °C**. **Conjugate** (1:101 diluted CONJ) **is not stable and cannot be stored.**
- All other test reagents are ready-to-use. Test reagents can be used until the expiry date (see label) when stored at **2–8 °C**.

6. STORAGE AND PREPARATION OF SAMPLES

Sample Storage

ZFP is stable in undiluted serum for 12 months at -80 °C as well as for 8 weeks at -20 °C and for 1 day at 2–8 °C. ZFP is not stable at room temperature.

Sample preparation

Sample must be diluted immediately before starting the test (see chapter "Assay procedure").

7. ASSAY PROCEDURE

Principle of the test

This ELISA is designed for the quantitative determination of ZFP in serum samples.

This assay is based on the method of competitive ELISA. As a first preparation step, a biotinylated ZFP 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-ZFP antibodies. During the incubation, the free target antigen in the samples competes with the biotinylated ZFP tracer for the binding of the polyclonal anti-ZFP antibodies immobilised on the microtiter plate wells. The unbound components are removed by a washing step. During a second incubation step, peroxidase-labelled streptavidin, which binds to the biotinylated ZFP tracer, is added into each microtiter well. After a washing step to remove the unbound components, the peroxidase substrate tetramethylbenzidine (TMB) 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 ZFP concentration in the sample; this means, high ZFP concentration in the sample reduces the concentration of the biotinylated ZFP tracer bound to the immobilised anti-ZFP 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. ZFP, present in the patient samples, is determined directly from this curve.

Test procedure

Bring all **reagents and samples to room temperature** (15–30 °C) and mix well.

Mark the positions of standards/controls/samples on a protocol sheet.

Take as many microtiter strips as needed from the kit. Store unused strips together with the desiccant bag in the closed aluminium packaging at 2–8 ° C. Strips are stable until 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.

Sample dilution

1.	Pipet each **25 µl** of **serum samples** in the respectively labelled reaction tubes.
2.	Add **475 µl** of **dilution buffer** to each sample. This results in a dilution factor of 20.

Preparation of standards, controls and diluted samples

3.	Transfer **150 µl** of each **standard**, **control** or **diluted sample** in the correspondingly labelled reaction tubes and add **150 µl** of **tracer.** Vortex well and use promptly in the test.

Important:

Carry out the **addition of tracer simultaneously with standards, controls and diluted samples** in order to ensure equal treatment.

Standards, controls and samples are now ready for use in the test.

4.	Add each **100 µl of the prepared standards/controls/samples** into the respective wells.
5.	Cover the strips and incubate for **1 hour shaking** on a horizontal shaker at 550 rpm with an orbit of 2 mm at room temperature (15–30 °C).
6.	Discard the content of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
7.	Add **100 µl conjugate** (diluted CONJ) into each well.

8.	Cover the strips and incubate for **1 hour shaking** on a horizontal shaker at 550 rpm with an orbit of 2 mm at room temperature (15–30 °C).
9.	Discard the content of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
10.	Add **100 µl substrate** (SUB) into each well.
11.	Incubate for **10–20 minutes** at room temperature (15–30 °C)* **in the dark**.
12.	Add **100 µl stop solution** (STOP) into each well and mix well.
13.	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 a 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.

Serum samples

The obtained results have to be multiplied by the **dilution factor of 20** to get the actual concentrations.

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

9. LIMITATIONS

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

Samples with concentrations lower than the measurement (see definition below) range 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*

LoB see chapter "Performance characteristics".

10. QUALITY CONTROL

Immundiagnostik AG 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 AG studies of serum samples of apparently healthy persons (n = 40), a median value of 34 ng/ml (± 14 ng/ml) was estimated.

We recommend each laboratory to establish its own reference range.

Establish own reference ranges for plasma samples.

11. PERFORMANCE CHARACTERISTICS

Accuracy – Precision

Repeatability (Intra-Assay); n = 40

The repeatability was assessed with 2 serum samples under **constant** parameters (same operator, instrument, day and kit lot).

Sample	Mean value [ng/ml]	CV [%]
1	43.90	3.5
2	38.38	6.0

Reproducibility (Inter-Assay); n = 25

The reproducibility was assessed with 2 serum samples under **varying** parameters (different operators, instruments, days and kit lots).

Sample	Mean value [ng/ml]	CV [%]
1	41.13	7.7
2	46.15	8.3

Accuracy – Trueness

The trueness states the closeness of the agreement between the result of a measurement and the true value of the measurand. Therefore, ZFP spikes with known concentrations were added to 3 different serum samples.

Sample [ng/ml]	Spike [ng/ml]	Expected [ng/ml]	Obtained [ng/ml]	Recovery [%]
10.788	51.58	62.469	66.331	106.2
	37.71	48.494	50.259	103.6
	26.64	37.430	34.469	92.1
12.428	51.58	64.109	70.666	110.2
	37.71	50.134	50.883	101.5
	26.64	39.070	33.032	84.5
13.372	51.58	65.053	70.547	108.4
	37.71	51.078	53.548	104.8
	26.64	40.014	34.266	85.6

Linearity

The linearity states the ability of a method to provide results proportional to the concentration of analyte in the test sample within a given range. This was assessed according to CLSI guideline EP6-A with a serial dilution of 2 different serum samples.

For ZFP in serum, the method has been demonstrated to be linear from 3.03–40.25 ng/ml, showing a non-linear behaviour of less than ± 20 % in this interval for concentrations greater than the Limit of Quantitation.

Sample	Dilution	Expected [ng/ml]	Obtained [ng/ml]	Recovery [%]
A	1:20	36.69	36.69	100.00
	1:40	18.34	19.02	103.70
	1:80	9.17	9.92	108.17
	1:160	4.59	5.91	128.79
B	1:20	40.25	40.25	100.00
	1:40	20.13	20.41	101.41
	1:80	10.06	10.90	108.29
	1:160	5.03	5.64	112.09
	1:320	2.52	3.03	120.52

Analytical sensitivity

The following values have been estimated based on the concentrations of the standard without considering possibly used sample dilution factors

Limit of blank, LoB 0.140 ng/ml

Limit of detection, LoD 0.183 ng/ml

Limit of quantitation, LoQ 0.183 ng/ml

The evaluation was performed according to the CLSI guideline EP17-A2. The specified accuracy goal for the LoQ was 20 % CV.

Analytical specificity

The specificity of the antibody was tested by measuring the cross-reactivity against human haptoglobin. There was no cross-reactivity observed.

Substance tested	Concentration added	Concentration obtained	Conclusion
Human haptoglobin	2.9 mg/ml	< 0.02 ng/ml	< LoB

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 or ProClin are hazardous to health and the environment. Substrates for enzymatic colour reactions may also cause skin and/or respiratory irritation. Any contact with the substances must be avoided. Further safety information can be found in the safety data sheet, which is available from Immundiagnostik AG on request.
- The 10x Wash buffer concentrate (WASHBUF) contains surfactants which may cause severe eye irritation in case of eye contact.

  Warning: Causes serious eye irritation. **IF IN EYES:** Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If eye irritation persists: get medical Advice/attention.
- 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, even if they are of the same batch.
- Control samples should be analysed 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 to 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

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Used symbols:


Temperature limitation

Catalogue number

In Vitro Diagnostic Medical Device

To be used with


Manufacturer

Contains sufficient for <n> tests

Lot number


Use by


Attention


Consult instructions for use

Consult specification data sheet


Irritant