

IDK[®] Zonulin ELISA

*For the determination of
zonulin family peptides (ZFP) in stool*

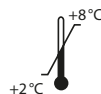
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1. INTENDED USE

This ELISA is intended for the quantitative determination of zonulin family peptides (ZFP) in stool. For research use only. Not for use in diagnostic procedures.

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.

The polyclonal antibody 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 5600	K 5600.20
KR5600	PLATE	Microtiter plate, pre-coated	12 x 8 wells	20 x 12 x 8 wells
KR0001.C.100	WASHBUF	Wash buffer concentrate, 10x	2 x 100 ml	40 x 100 ml
KR5600	DIL	Dilution buffer concentrate, 2.5 x	2 x 100 ml	20 x 100 ml
KR5600	TRACER	Tracer concentrate (biotinylated ZFP)	1 x 300 µl	20 x 300 µl
KR5600	CONJ	Conjugate concentrate (peroxidase-labelled streptavidin)	1 x 200 µl	20 x 200 µl

Cat. No.	Label	Kit components	Quantity for cat. no.	
			K 5600	K 5600.20
KR5600	STD	Standards, lyophilised (see specification for concentrations)	4 x 5 vials	25 x 5 vials
KR5600	CTRL1	Control, lyophilised (see specification for range)	4 x 1 vial	25 x 1 vial
KR5600	CTRL2	Control, lyophilised (see specification for range)	4 x 1 vial	25 x 1 vial
KR0002.15	SUB	Substrate (tetramethylbenzidine), ready-to-use	1 x 15 ml	20 x 15 ml
KR0003.15	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*
- Stool sample application system such as Cat. No.: K 6998SAS
- Calibrated precision pipettors and 10–1000 µl single-use tips
- Foil to cover the microtiter plate
- Horizontal microtiter plate shaker (available via Immundiagnostik AG upon request)
- Multi-channel pipets or repeater pipets
- Vortex
- Standard single-use laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader

* 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)** should 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** 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 1 month**.
- **Preparation of the dilution buffer:** The **dilution buffer concentrate (DIL)** should be diluted with **ultrapure water 1:2.5** before use (100 ml DIL + 150 ml ultrapure water), mix well. Crystals can occur due to high salt concentration in the concentrate. Before dilution, the crystals have to be redissolved in a water bath at 37°C. 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 1 month**.
- **Preparation of standards and controls:** The **lyophilised standards (STD)** and **controls (CTRL)** are stable at **2–8°C** until the expiry date stated on the label. **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** (150 µl TRACER + 15 ml dilution buffer) immediately before use. The TRACER is stable at **2–8°C** until the 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)** has to be diluted **1:101** in dilution buffer (100 µl CONJ + 10 ml dilution buffer) immediately before use. The CONJ is stable at **2–8°C** until the 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) when stored at **2–8°C**.

6. STORAGE AND PREPARATION OF SAMPLES

Sample stability

ZFP is stable in **raw stool** for 3 months at -20 °C as well as for 4 days at 2-8 °C and room temperature.

ZFP is stable in **stool extracts** for 7 days at -20 °C as well as for 4 days at 2-8 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 dilution 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 stool sample tube** with **0.75 ml of dilution buffer** before using it with the sample. Important: Allow the dilution buffer to reach room temperature.
- c) Unscrew the tube (yellow part of cap) to open. Insert the yellow 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 dilution 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**

7. PREPARATION OF THE ASSAY

Prior to use, allow **all reagents** and **samples** to come 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.

We recommend to carry out the tests in duplicate.

Preparation of standards, controls and diluted samples

Transfer **150 µl** of each **standard, control** or **stool extract** 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 stool extracts in order to ensure equal treatment.

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

8. ASSAY PROCEDURE

Principle of the test

This assay is based on the method of competitive ELISA. As a first preparation step, biotinylated ZFP 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 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, 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 ZFP concentration in the sample; this means, high ZFP concentration in the sample reduces the concentration of the biotinylated ZFP 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.

Test procedure

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.

1.	Add each 100 µl of the prepared standards/controls/samples into the respective wells.
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.	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.
4.	Add 100 µl conjugate (diluted CONJ) 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.	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 substrate (SUB) into each well.
8.	Incubate for 10–20 min* at room temperature (15–30 °C) in the dark .
9.	Add 100 µl stop solution (STOP) into each well and mix well.

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 the highest standard exceeds the range of the photometer, absorption must be measured immediately at 405 nm against 620 nm as a reference.
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* The intensity of the colour change is temperature sensitive. We recommend observing the colour change and stopping the reaction upon good differentiation.

9. 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 results have to be multiplied by the **dilution factor of 50** to get the actual concentrations.

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

10. 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 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

LoB see chapter "Performance Characteristics".

11. 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 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

We recommend each laboratory to establish its own reference range.

12. PERFORMANCE CHARACTERISTICS

Accuracy – Precision

Repeatability (Intra-Assay); n = 24

The repeatability was assessed with 3 stool samples under **constant** parameters (same operator, measurement system, day and kit lot).

Sample	Mean value [ng/ml]	CV [%]
1	117.68	6.4
2	130.91	6.0
3	38.28	3.3

Reproducibility (Inter-Assay); n = 25

The reproducibility was assessed with 3 stool samples under **varying** parameters (different operators, measurement systems, days and kit lots).

Sample	Mean value [ng/ml]	CV [%]
1	74.01	13.1
2	53.30	17.6
3	61.98	18.3

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

0.118 ng/ml

12. PRECAUTIONS

- All reagents in the kit package are for research 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 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












- The guidelines for 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 sent to Immundiagnostik AG along with a written complaint.

15. REFERENCES

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Used symbols:

	Temperature limitation		Catalogue Number
	For research use only		To be used with
	Manufacturer		Contains sufficient for <n> tests
	Lot number		Use by
	Attention		Consult instructions for use
	Consult specification data sheet		