Human Zonulin ELISA Kit

PromoKine

Instruction Manual

Cat. No. PK-EL-K5600

Contents

Intended Use

This PromoCell ELISA is intended for the quantitative determination of Zonulin/Zonulin Family Peptides (ZFP) in serum. For research use only.

Introduction

Zonulin is a novel human protein analogue to the Vibrio cholerae derived Zonula ccludens toxin, 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.

Dr. Fasano and his co-workers found out that the zonulin-zonulin-receptorsystem 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 eating a gluten-free diet.

Concerning the autoimmune type 1 diabetes, in experiments with rats 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.

Principle of the Test

This enzyme immuno assay can be used for the quantitative determination of Zonulin/Zonulin Family Peptides (ZFP) in serum samples.
The assay is based on the method of competitive enzyme linked immunoassays. As a first preparation step, a biotinylated zonulin tracer is added to the samples, standards and controls. Afterwards, aliquots of the treated preparations 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. During a second incubation step, a streptavidin-labeled-peroxidase antibody, which binds to the biotinylated zonulin tracer, is added into each microtiter well. After washing the unbound components, the peroxidase substrate tetramethylbenzidine (TMB) is added. Finally, the enzymatic reaction is terminated by an acidic stop solution. The color changes from blue to yellow and the absorbance is measured in the photometer at 450 nm. The intensity of the yellow color 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. Zonulin present in patient samples is determined directly from this curve.

Material Supplied

Label	Kit Components	Quantity
MTP	Microtiter plate, precoated	12 x 8 wells
WASHBUF	BM-Wash Buffer Concentrate 10 x	2 x 100 ml
DIL	Dilution Buffer, ready to use	1 x 100 ml
TRACER	Tracer Biotinylated Zonulin	1 x 300 µl
CONJ	Conjugate; peroxidase-labeled streptavidin	1 x 200 µl
STD	Standards (lyophilized)	4 x 5 vials
CTRL 1	Control 1 (lyophilized)	4 x 1 vial
CTRL 2	Control 2 (lyophilized)	4 x 1 vial
SUB	TMB substrate, ready to use	1 x 15 ml
STOP	ELISA stop solution, ready to use	1 x 15 ml

Material Required but not Supplied

- Ultra-pure water*
- Precision pipettors and disposable tips to deliver 5-1000 µl
- Foil to cover the microtiter plate
- Horizontal microtiter plate shaker
- A multi-channel dispenser or repeating dispenser
- Centrifuge capable of 3,000 x g
- Eppendorf-cups, 1,5 ml
- 15 ml tubes (e. g. Falcon)
- Vortex-Mixer
- Standard laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader at 450 or 405 nm (reference wave length 620 or 690 nm)

* We recommend the use of Ultra Pure Water (Water Type 1; ISO3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity < 0.055 µS/cm at 25°C (≥18.2 MΩ cm).

Preparation and Storage of Reagents

- To run assay more than once ensure that reagents are stored at conditions stated on the label. **Prepare only the appropriate amount necessary for each assay**. 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.
- The **WASHBUF** (BM wash buffer concentrate) should be diluted with ultra-pure 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 37°C in a water bath before dilution of the buffer solutions. The WASHBUF is stable at 2-8°C until the expiry date stated on the label. Diluted buffer solution can be stored in a closed flask at 2-8°C for one month.
- The lyophilized **STD (standards) and CTRL (controls)** are stable at 2-8°C until the expiry date stated on the label. Lyophilized STDs (standards) and CTRLs (controls) have to be reconstituted with ultra-pure water (volume and concentration see CoA). Allow the vial content to dissolve for at least 10 minutes and mix thoroughly by gentle inversion to insure complete reconstitution. **Reconstituted standards and controls are not stable and cannot be stored.**
- The **TRACER** (biotinylated Zonulin-Tracer) must be diluted 1:101 in **DIL** (150 µl TRACER + 15 ml dilution buffer). The TRACER (biotinylated Zonulin-Tracer) is stable at 2 -8 °C until expiry date given on the label. **Diluted TRACER is not stable and cannot be stored.**
- The **CONJ (Conjugate)** must be diluted 1:101 in WASHBUF (100 µl CONJ + 10 ml wash buffer). The CONJ (Conjugate) is stable at 2 -8 °C until expiry date given on the label. **Diluted CONJ is not stable and can not be stored**.
- All other test reagents are ready to use and stable until the expiry date (see respective labels) when stored at 2-8°C.

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.

Specimen Collection and Preparation

Serum

Store samples until use at -20 °C.

Note: Zonulin/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. Zonulin is not stable at room temperature.

1. Pipette **25 µl of serum sample** in the respective labeled Eppendorf-cups.
2. Add **475 µl of dilution buffer** (DIL) to each sample, vortex well. This results in a **dilution factor of 20.**
3. Then pipette **150 µl of diluted Sample** in labeled Eppendorf-cups and add **150 µl of diluted TRACER** to each sample, vortex well.

Preparation of Standards and Controls

Transfer **150 µl of STD or CTRL** in the corresponding labelled reaction tube, add 150 µl of **diluted Tracer** and mix well by vortexing.

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 to use and should be used promptly in the test.

Assay Procedure

Procedural Notes

- Do not interchange different lot numbers of any kit component within the same assay.
- 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.
- The assay should always be performed according the enclosed manual.

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 aluminium wrapper at 2–8 °C. Strips are stable until the expiry date stated on the label.

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

For automated ELISA processors, the given protocol may need to be adjusted according to the specific fearures of the respective automated platform. Please contact your suppler for details.

We recommend to carry out the tests in duplicate.

1. Add **100 µl** of the **STD** (standards), **CTRL** (controls) or **samples** into each respective well in duplicate.
2. 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).
3. Discard the contents of each well. Wash the microtiter plate **5 times** with **250 µl of diluted wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the inverted microtiter plate on absorbent paper.
4. Add **100 µl** of **CONJ** (conjugate) into each well.
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. Wash the microtiter plate **5 times** with **250 µl of diluted wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the inverted microtiter plate on absorbent paper.
7. Add **100 µl** of **SUB** (substrate solution) into each well.
8. Incubate for **10-20 minutes** at room temperature (15-30°C)* **in the dark**.
9. Add **100 µl** of **STOP** (stop solution) into each well and mix well.

10. Determine absorption **immediately** with an ELISA reader at **450 nm** against 620 nm (or 690 nm). If no reference wavelength is available, read only at 450 nm. If the extinction of a sample or the standards **(STD)** 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 colourchange and stopping the reaction upon good differentiation.

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 calibrator 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 logarithmic abscissa for the concentration. When using a logarithmic abscissa, the zero calibrator must be specified with a value less than 1 (e.g. 0.01).

The plausibility of the pairs of values should be examined before the automatic evaluation of the results. If this option is not available with the used program, a control of the paired values should be done manually.

Serum samples

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

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

Limitations

Samples with concentrations above the measurement range must be further diluted and re-assayed. Please consider this greater dilution when calculating the results.
Samples with concentrations lower than the measurement 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

Quality Control

We recommend 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 internal 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.

Performance Characteristics

Precision and Reproducibility

Intra-Assay n= 40

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

Sample	Zonulin [ng/ml]	VK [%]
1	43.90	3.5
2	38.38	6.0

Inter-Assay n= 25

The repeatability was assessed with 2 serum samples under varying parameters (different operators, measurement systems, days and kit lots).

Sample	Zonulin [ng/ml]	VK [%]
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, zonulin 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 zonulin 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]	Measured [ng/ml]	Recovery [%]
A	1:20	36.69	36.69	100
	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
	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

References

- Wang W, Uzzau S, Goldblum SE, Fasano A. Human zonulin, a potential modulator of intestinal tight junctions. J Cell Sci 2000; 113 Pt 24: 4435-40.
- Fasano A. Intestinal zonulin: open sesame! Gut 2001; 49: 159-62.
- Fasano A, Not T, Wang W et al. Zonulin, a newly discovered modulator of intestinal permeability, and its expression in celiac disease. Lancet 2000; 355(9214): 1518-19.
- Thomas KE, Sapone A, Fasano A, Vogel SN. Gliadin stimulation of murine macrophage inflammatory gene expression and intestinal permeability are MyD88-dependent: role of the innate immune response in Celiac disease. J Immunol 2006; 176: 2512-21.
- Freemark M, Lynne LL. Screening for Celiac Disease in Children with Type 1 Diabetes. Diabetes Care 2003; 26: 1932-39.
- Lazzarotto F, Basso D, Plebani M et al. Celiac Disease and Type 1 Diabetes. Diabetes Care 2003; 26: 248-49.
- Watts T, Berti I, Sapone A et al. Role of the intestinal tight junction modulator zonulin in the pathogenesis of type 1 diabetes in BB diabeticprone rats. Proc Nat Acad Sci USA 2005; 102(8): 2916-21.
- Sapone A, de Magistris L, Pietzak M, Clemente MG, Tripathi A, Cucca F, Lampis R, Kryszak D, Carteni M, Generoso M, Iafusco D, Prisco F, Laghi F, Riegler G, Carratu R, Counts D, Fasano A. Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. Diabetes 2006; 55: 1443-9.
- De Magistris MT. Zonula occludens toxin as a new promising adjuvant for mucosal vaccines. Vaccine 2006;24 Suppl 2: S2-60-1.

General Notes on the Test and Test Procedure

- All reagents in the test package are for research use only.
- 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.
- The assay should always be performed according the enclosed manual.
- The quality control guidelines should be observed.
- 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. PromoCell can therefore not be held responsible for any damage resulting from wrong use.
- Warranty claims and complaints in respect of deficiencies must be logged within 14 days after receipt of the product. The product shall be sent to PromoCell along with a written complaint.

Ordering Information

Product Name	Product Description	Size	Catalog Number
Zonulin ELISA Kit, human	Human Zonulin ELISA Kit	96 Tests	PK-EL-K5600

For in vitro research use only.
Not for diagnostic or therapeutic procedures.

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