Zonulin Stool ELISA

For the quantitative determination of zonulin family peptides (ZFP) in human stool.

For Research Use Only. Not For Use in Diagnostic Procedures.

Catalog Number: 30-ZONHU-E01, E10
Size: 96 Wells, 10 x 96 wells
Version: 2023-07-25 – ALPCO 5.0

INTENDED USE

The Zonulin Stool ELISA is intended for the quantitative determination of zonulin family peptides (ZFP) in human stool samples. For Research Use Only. Not for use in diagnostic procedures.

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 induce 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).

PRINCIPLE OF THE ASSAY

This assay is based on the method of a competitive ELISA. As a first preparation step, biotinylated zonulin family peptide 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 family peptide antibodies. During the incubation, the free target antigen in the samples competes with the biotinylated zonulin family peptides for the binding of the polyclonal anti-zonulin family peptide 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 family peptides, 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 color changes from blue to yellow and the absorbance is measured in the photometer at 450 nm. The intensity of the yellow color is inversely proportional to the zonulin family peptides concentration in the sample. A dose response curve of absorbance units (optical density, OD at 450 nm) versus concentration is generated using the values obtained from the standards.

MATERIALS SUPPLIED

Product: 30-ZONHU-E01

Component	Quantity	Preparation
Zonulin Microplate (96 wells)	12 x 8 strips	Ready to use
Standards (1-5) *	2 vials each	Lyophilized
Control Levels* 1 and 2	2 vials each	Lyophilized
Wash Buffer Concentrate	2 x 100 mL	10X
Dilution Buffer Concentrate	2 x 100 mL	2.5X
Tracer Concentrate (Biotinylated ZFP)	1 x 300 µL	101X
Conjugate Concentrate	1 x 200 µL	101X
TMB Substrate	1 x 15 mL	Ready to use
Stop Solution	1 x 15 mL	Ready to use

*Consult Certificate of Analysis for concentrations.

Product: 30-ZONHU-E10

Component	Quantity	Preparation
Zonulin Microplate (96 wells)	10 Plates: each	Ready to use

	12 x 8 strips	
Standards (1-5) *	20 vials each	Lyophilized
Control Levels* 1 and 2	20 vials each	Lyophilized
Wash Buffer Concentrate	20 x 100 mL	10X
Dilution Buffer Concentrate	20 x 100 mL	2.5X
Tracer Concentrate (Biotinylated ZFP)	10 x 300 µL	101X
Conjugate Concentrate	10 x 200 µL	101X
TMB Substrate	10 x 15 mL	Ready to use
Stop Solution	10 x 15 mL	Ready to use

*Consult Certificate of Analysis for concentrations.

MATERIALS REQUIRED BUT NOT SUPPLIED

- Precision pipettes for dispensing up to 1000 µL (with disposable tips)
- Repeating or multi-channel pipette for dispensing up to 1000 µL
- Volumetric containers and pipettes for reagent preparation
- Distilled/Deionized water for reagent preparation
- Microplate washer or wash bottle
- Microplate shaker capable of 350 rpm
- Microplate reader
- Centrifuge (1,000 x g to 3,000 x g) for sample preparation
- Vortex for sample preparation
- Foil to cover the microplate
- Timer

PRECAUTIONS

1. Kit reagents contain sodium azide or Proclin as bactericides. Sodium azide and Proclin are hazardous to health and the environment. Substrates for the enzymatic color 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 ALPCO upon request.
2. The stop solution consists of diluted sulfuric 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 breathe vapor and avoid inhalation.
3. The 10x Wash Buffer concentrate 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.
4. Avoid direct contact with skin.
5. This product is not for internal use.
6. Avoid eating, drinking, or smoking when using this product.

Do not pipette any reagents by mouth.

STORAGE CONDITIONS

The kit should be stored at 2-8°C. The properly stored kit is stable until the expiration date on the box label.

SAMPLE HANDLING

Stool samples are appropriate for use in this assay.

The sample stability is as follows:
Raw stool: 4 days at 2-8°C or room temperature and 3 months at -20°C.
Stool extracts (1:50): 4 days at 2-8°C and 7 days at -20°C.

Extraction of the Stool Sample: 1X working dilution buffer is used as a sample extraction buffer. It is recommended to perform the following sample preparation:

1. The raw stool sample must be thawed. For particularly heterogeneous samples it is recommended to perform a mechanical homogenization using an applicator, inoculation loop or similar device.
2. Fill the empty tube (or empty Easy Extraction Device) with 0.75 mL of prepared 1x working dilution buffer before using it with the sample. Important: Allow the dilution buffer to reach room temperature.
3. If using the Easy Extraction Device, unscrew the tube (yellow part of cap) to open. Insert yellow dipstick into sample at three different locations. The lower part of the dipstick has notches which must be covered completely with stool after inserting it into the sample. Place dipstick back into the tube only once. 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. If using a manual method, weigh and add 15mg of sample.
4. 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 approximately 10 minutes improves the result.
5. Allow sample to stand for approximately 10 minutes until sediment has settled. Floating material like shells of grains can be ignored.
6. 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. This will result in a dilution of 1:50.

Preparation of standards, controls, and diluted samples:
Pipet 150 µL of each standard, control, or diluted stool extract into the correspondingly labeled reaction tubes and add 150 µL of prepared tracer (*See Reagent Preparation*). Vortex well and use promptly in the assay. Important: Add tracer simultaneously with standards, controls, and stool extracts to ensure equal treatment.

REAGENT PREPARATION

To run the assay more than once, ensure that the reagents are stored at the conditions stated on the label. Prepare only the appropriate amount needed for each run. All reagents must be equilibrated to room temperature prior to preparation and subsequent use in the assay. Prepare enough reagents for only the number of strips used. Reagents with a volume less than 100 µL should be centrifuged before use to avoid loss of volume. If stored properly, the kit can be used up to 2 times within the expiry date stated on the label.

Controls (Levels 1 and 2) and Standards are provided in a lyophilized form. The lyophilized standards and controls are stable at 2-8°C until the expiry date stated on the label. Reconstitute according to the Certificate of Analysis. Reconstituted controls and standards are not stable and cannot be stored.

Wash Buffer Concentrate (10X) must be diluted with distilled or deionized water 1:10 before use (100 mL wash buffer concentrate + 900 mL distilled water) and mixed well. Crystals could occur due to high salt concentration in the concentrate. Before dilution, the crystals must be dissolved at room temperature or in a water bath at 37 °C. The wash buffer concentrate is stable at 2–8 °C until the expiry date stated on the label. 1X working wash buffer (diluted 1:10) can be stored in a closed flask at 2–8 °C for 1 month.

Dilution Buffer Concentrate (2.5X) must be diluted with distilled or deionized water 1:2.5 before use (100 mL Dilution Buffer + 150 mL distilled water) and mixed well. Crystals could occur due to high salt concentration in the concentrate. Before dilution, the crystals must be dissolved at 37°C in a water bath. The dilution buffer concentrate is stable at 2–8 °C until the expiry date stated on the label. 1X working dilution buffer (diluted 1:2.5) can be stored in a closed flask at 2–8 °C for 1 month.

Conjugate Concentrate (101X) must be diluted 1:101 in 1X working dilution buffer immediately before use (100 µL conjugate concentrate + 10 mL 1X working dilution buffer). The concentrate is stable at 2–8°C until the expiry date stated on the label. Diluted conjugate is not stable and cannot be stored.

Tracer (101X) must be diluted 1:101 in 1X working dilution buffer (150 µL tracer + 15 mL 1X working dilution buffer) immediately before use. The tracer is stable at 2–8°C until the expiry date stated on the label. Diluted tracer 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.

QUALITY CONTROL

Control samples should be analyzed 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 samples are outside the acceptable limits.

ASSAY PROCEDURE

All reagents and microplate strips (while sealed in foil pouch) should be equilibrated to room temperature (15 - 30 °C) prior to use. Gently mix all reagents before use. A standard curve must be performed with each assay run and with each microplate if more than one is used at a time. All standards, controls, and samples should be run in duplicate. Mark the positions of standards, controls, and samples on a protocol sheet before beginning the assay.

Take as many microtiter strips as needed from the kit. Store unused strips together with the desiccant bag in the closed aluminum 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 ALPCO.

1. Add 100 µL of the prepared standards, controls, or samples (premixed with tracer) 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 contents of each well. Wash the microtiter plate 5 times with 250 µL 1X working wash buffer. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
4. Add 100 µL of 1X working conjugate into each well *(See Reagent Preparation section)*.
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 contents of each well. Wash the microtiter plate 5 times with 250 µL 1X working wash buffer. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
7. Add 100 µL of TMB Substrate into each well.
8. Incubate for 10–20* minutes at room temperature (15–30°C) in the dark.
9. Add 100 µL of 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) 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 color change is temperature sensitive. It is recommended to observe the color change and to stop the reaction upon good differentiation.

CALCULATION OF RESULTS

The following algorithms can be used alternatively to calculate the results. It is recommended to use 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: It is recommended to use a linear ordinate for the optical density and a linear abscissa for the concentration.
3. Spline algorithm: It is recommended to use a linear ordinate for the optical density and a linear abscissa for the concentration.

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 program used, a comparison of the duplicate values should be performed manually.

Stool Samples

The obtained zonulin family peptides levels of the stool samples must be multiplied by the dilution factor of 50 to obtain the actual concentrations.

If another dilution factor has been used, multiply the obtained result by the dilution factor used.

LIMITATIONS

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:
Limit of blank (LoB) × sample dilution factor to be used

PERFORMANCE CHARACTERISTICS

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

Precision: Within run (intra-assay) variation (n = 24)

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

Sample	Zonulin (ng/mL)	CV (%)
1	117.7	6.4
2	130.9	6.0
3	38.3	3.3

Precision: Between run (inter-assay) variation (n = 25)

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

Sample	Zonulin (ng/mL)	CV (%)
1	74.0	13.1
2	53.3	17.6
3	62.0	18.3

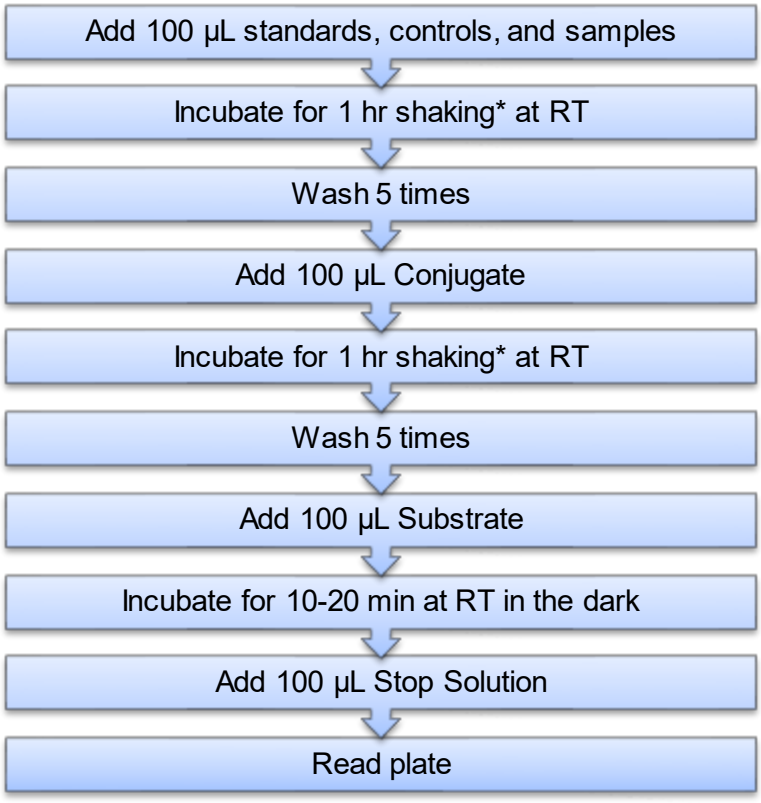
TECHNICAL HINTS

- Do not interchange different lot numbers of any kit component within the same assay. Furthermore, it is not recommended to assemble wells of different microtiter plates for analysis, even if they are of the same batch.
- Control samples should be analyzed with each run.
- Reagents should not be used beyond the expiration date stated on the kit label.
- Substrate solution should remain colorless 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.

GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- The guidelines for laboratories should be followed.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the vendor. Any variation of the test procedure, which is not coordinated with the vendor, may influence the results of the test.
- Claims or complaints regarding deficiencies must be logged within 14 days of receipt of the product.

SHORT ASSAY PROTOCOL



Total Time = 2 hours 20 minutes
Recommended Plate Shaker Settings: 350 rpm with orbit of 2 mm

SUGGESTED PLATE LAYOUT

Below is a suggested plate layout for running standards, controls, and up to 41 samples in duplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 1	2	2	10	10	18	18	26	26	34	34
B	Std 2	Std 2	3	3	11	11	19	19	27	27	35	35
C	Std 3	Std 3	4	4	12	12	20	20	28	28	36	36
D	Std 4	Std 4	5	5	13	13	21	21	29	29	37	37
E	Std 5	Std 5	6	6	14	14	22	22	30	30	38	38
F	Ctrl 1	Ctrl 1	7	7	15	15	23	23	31	31	39	39
G	Ctrl 2	Ctrl 2	8	8	16	16	24	24	32	32	40	40
H	1	1	9	9	17	17	25	25	33	33	41	41

Std= Standard
Ctrl = Control
Numbered wells = Samples