Product information

User´s Manual

β-Defensin 2 ELISA

For the determination of β-Defensin 2 in stool.

For research use only, not for use in diagnostic procedures.

REF	**ID09021**
Σ	**96**
Storage:	**2-8°C**

Manufactured for:
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1. INTENDED USE

This assay is intended for the determination of β-defensin 2 in stool. For research use only, not for use in diagnostic procedures.

2. INTRODUCTION

The ß-defensins are an integral part of the congenital immune system and contribute with their antimicrobial effect to the barrier function of intestinal epithelial cells.

Defensins exert a variable degree of antimicrobial activity against bacteria, fungi, and some enveloped viruses. Vertebrate defensins are classified as alpha- or beta-defensins, based on their pattern of disulfide bridges. Nine human defensins of epithelial origin have been found, three of them being ß-defensins (HBD-1, -2 and -3). The expression of ß-defensins is induced by the pro-inflammatory cytokines and also through microorganisms (e.g. *F. coli*, *H. pylori* or *P. aeruginosa*).

A ß-defensin-2 deficiency can, for example, be observed in the intestinal mucous of subjects with Crohn's disease. The defense system of the mucous membrane is therefore restricted and allows an increased invasion of bacteria, which could possibly lead to a typical infection in Crohn's disease subjects.

Whether the ß-defensin-2 deficiency could even play a role in the development of Crohn's disease is currently being researched. As is the possibility that it is the probiotic bacterium, which produces ß-defensin.

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity
K 6500MTP	PLATE	Holder with precoated strips	12 x 8 wells
K 6500WP	WASHBUF	ELISA wash buffer concentrate 10x	2 x 100 ml
K 6500K	CONJ	Conjugate concentrate, (Streptavidin, HRP-conjugated)	200 µl
K 6500ST	STD	Standards, lyophilized	2 x 5 vials
K 6500SV	STDBUF	Standard dilution buffer	20 ml
K 6500KO1	CTRL	Control, lyophilized	2 x 1 vial
K 6500KO2	CTRL	Control, lyophilized	2 x 1 vial

Cat. No.	Label	Kit components	Quantity
K 6500EP	IDK Extract®	Extraction buffer concentrate *IDK Extract*® 2.5x	2 x 100 ml
K 6500TMB	SUB	TMB substrate (tetramethylbenzidine), ready to use	15 ml
K 6500AC	STOP	ELISA stop solution, ready to use	15 ml

4. MATERIAL REQUIRED BUT NOT SUPPLIED

- Ultra pure water*
- Laboratory balance
- Calibrated precision pipettors and 10–1000 µl tips
- Foil to cover the microtiter plate
- Horizontal microtiter plate shaker
- Multi-channel pipets or repeater pipets
- Centrifuge, 3000 *g*
- Vortex
- Standard laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader (required filters see chapter 7)

* IBL-America 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. STORAGE AND PREPARATION 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.
- The **ELISA wash buffer concentrate** (WASHBUF) should be diluted **1:10 in ultra pure water** before use (100 ml concentrate + 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 37 °C before dilution of the buffer solutions. The **buffer concentrate** is stable at **2–8 °C** until the expiry date stated on the label. **Diluted buffer solution** (wash buffer) can be stored in a closed flask at **2–8 °C for one month**.

- The **extraction buffer concentrate *IDK Extract*®** must be diluted with ultra pure water **1:2.5** before use (100 ml concentrate + 150 ml ultra pure water), mix well. Crystals could occur due to high salt concentration in the stock solutions. Before dilution, the crystals must be redissolved at 37°C in a water bath. The **extraction buffer concentrate *IDK Extract*®** is stable at **2 - 8 °C** until the expiry date stated on the label. Diluted **buffer solution** (extraction buffer) can be stored in a closed flask at **2 - 8 °C for three months**.
- The lyophilized **STD** (standards) and **CTRL** (controls) must be reconstituted with **500 µl** of **STDBUF** (standard dilution buffer). Allow the vial content to dissolve for 10 minutes and mix thoroughly by gentle inversion to insure complete reconstitution. **Reconstituted standards and controls** can be stored for **two weeks at 2–8 °C** or for **four weeks at -20 °C** and be used **once after thawing.**
- The **conjugate concentrate** (CONJ) must be diluted **1:101 in wash buffer** (100 µl CONJ + 10 ml wash 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.**
- 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. STORAGE AND PREPARATION OF SAMPLES

Sample stability

Raw stool: 48 hours at 2–8 °C, at least 4 weeks at -20 °C

Stool extracts (1:100): 3 days at room temperature (15–30 °C), 7 days at 2–8 °C or 7 days at -20 °C, maximum 2 freeze-thaw cycles

Extraction of the stool samples

Diluted extraction buffer *IDK Extract*® is used as a sample extraction buffer. We recommend the following sample preparation:

Stool Sample Application System (SAS) (Cat. No.: K6998SAS)

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 1.5 ml extraction buffer:

Applied amount of stool: 15 mg

Buffer Volume: 1.5 ml

Dilution Factor: 1:100

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 homogenization using an applicator, inoculation loop or similar device.

b) Fill the **empty sample tube** with **1.5 ml** of ready to use *IDK Extract*® extraction buffer before using it with the sample. Important: Allow the extraction buffer to reach room temperature.

c) Unscrew the tube (orange 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 I: 1:100

Dilution of samples

Dilute the supernatant of the extraction (**dilution step I**) **1:2 with wash buffer**, for example: 300 µl supernatant (dilution I) + 300 µl wash buffer = 1:2 (**dilution step II**). This results in a final dilution of **1:200**.

For analysis, pipet **100 µl of dilution step II** per well.

7. ASSAY PROCEDURE

Principle of the test

The 13-defensin 2 in standards and samples is bound to an available excess of polyclonal antibodies against 13-defensin 2, which are immobilized on the surface of the microtiter plate. After a washing step, to remove all interfering substances, the determination of bound 13-defensin 2 is carried out by adding a polyclonal anti 13-defensin 2 antibody, which is horseradish peroxidase labeled. 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 color changes from blue to yellow and the absorbance is measured in the photometer at 450 nm. The intensity of the yellow color proportional to the 13-defensin 2 concentration in the sample. A dose response curve of absorbance unit (optical density, OD at 450 nm) vs. concentration is generated using the values obtained from the standards.

Test procedure

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

Take as many **microtiter strips** as needed from kit. Store unused strips covered at 2–8 ° C. Strips are stable until expiry date stated on the label.

We recommend to carry out the tests in duplicate.

1.	Wash the precoated microtiter plate **5 x with 250 µl ELISA wash buffer**.
2.	Pipet **100 µl** of **STD** (standards), **CTRL** (controls) or **samples** into each well.
3.	Incubate for **1 hour at room temperature** (15–30 °C), shaking on a horizontal mixer.
4.	Decant the contents of the plate and wash the cavities **5 x with 250 µl of washing buffer** solution.
5.	Add **100 µl diluted CONJ** (conjugate solution).
6.	Incubate for **1 hour at room temperature** (15–30 °C), shaking on a horizontal mixer.
7.	Decant the contents of the plate and wash the cavities **5 x with 250 µl of washing buffer** solution.
8.	Add **100 µl of SUB** (TMB substrate) solution

9.	Incubate approximately for **10–20 minutes* at room temperature** (15–30 °C) until sufficient coloring is achieved.
10.	Add **100 µl of STOP** (stop solution) and mix shortly.
11.	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.

* The intensity of the color change is temperature sensitive. We recommend to observe the color change and to stop the reaction upon good differentiation.

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.

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 program used, the duplicate values should be evaluated manually.

Stool

To obtain the β-defensin 2 concentration in fecal samples, multiply the estimated value by the dilution factor **200** according to the sample preparation.

9. 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 determined.

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

IBL-America recommends the use of external controls for internal quality control, if possible.

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 sample are outside the acceptable limits.

Reference range

1 g stool is equivalent to 1 ml.

Stool (n = 101): 35 ng/ml

Based on studies of evidently healthy persons (n = 101) a mean value of 35 ng/ml stool was estimated. This value is consistent with the results published using the ß-Defensin ELISA Kit of IBL-America.

Stool (n = 23 healthy controls):	31.0 ± 15.4 ng/g stool[3]
Reference range in stool samples:	8 - 60 ng/ml stool[1]

We recommend each laboratory to establish its own reference range.

11. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

Intra-Assay (n = 20)

The reproducibility was calculated by measuring each of two samples 20 times by one technician within the same assay.

Sample	β-Defensin 2 [ng/mL]	CV [%]
1	17.7	4.1
2	81.4	3.0

Inter-Assay (n = 20)

The total reproducibility was independently measured by several different technicians on 3 different days.

Sample	β-Defensin 2 [ng/mL]	CV [%]
1	95.5	9.1
2	10.3	8.1

Dilution recovery

Two samples were extracted as given in the manual and serial diluted.

Sample	Dilution	β-Defensin 2 expected [ng/ml]	β-Defensin 2 measured [ng/ml]
A	1:200	-	39.8
	1:400	19.9	19.8
	1:800	10.0	10.7
	1:1600	5.0	5.6
B	1:200	-	218.8
	1:400	109.4	105.7
	1:800	54.7	50.6
	1:1600	27.4	24.7

Analytical Sensitivity

Limit of blank

The calculated detection limit (LoB; Limit of Blank) was set as B_0 + 1.645 * SD. Standard 1 (blank) was measured 60 times. The values were estimated in relation to the concentration of the calibration curve and resulted in

- a detection limit of 0.02 ng/ml without consideration of the sample dilution factor
- a detection limit of 2 ng/ml with consideration of the sample dilution factor

Limit of detection

The calculated detection limit (LoD; Limit of Detection) was set as LoB + 1.645 * SD. Standard 1 (blank) was measured 72 times. The values were estimated in relation to the concentration of the calibration curve and resulted in

- a detection limit of 0.06 ng/ml without consideration of the sample dilution factor
- a detection limit of 6 ng/ml with consideration of the sample dilution factor

12. PRECAUTIONS

- All reagents in the kit package are for research use only, not for use in diagnostic procedures.
- 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 color 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 breathe vapor 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 analyzed with each run.
- Reagents should not be used beyond the expiration date stated on 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 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.
- Quality control guidelines should be followed.
- 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. IBL-America 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 should be send to IBL-America along with a written complaint.

15. PUBLICATIONS USING K 6500 ELISA KIT

1. Döll, M., Hauss, R. & Spermezan, R. Immunmodulierende Wirkung von (1-3),(1-6)-beta-D-Glucan -- gezeigt an der Neopterin- und b-Defensin-Synthese. *Naturheilpraxis* **05**, 676–681 (2005).
2. Soto, E. et al. Human beta-defensin-2: a natural antimicrobial peptide present in amniotic fluid participates in the host response to microbial invasion of the am-

niotic cavity. *The journal of maternal-fetal & neonatal medicine* **20**, 15–22 (2007).

3. Langhorst, J. et al. Activated innate immune system in irritable bowel syndrome? *Gut* **56**, 1325–6 (2007).
4. Schwab, M. et al. The dietary histone deacetylase inhibitor sulforaphane induces human beta-defensin-2 in intestinal epithelial cells. *Immunology* **125**, 241–51 (2008).
5. Schwiertz, A., Huber, H. & Rusch, K. Human beta-defensin-2 levels in healthy individuals. *The American journal of gastroenterology* **104**, 2110; author reply 2110–1 (2009).
6. Langhorst, J. et al. Elevated human beta-defensin-2 levels indicate an activation of the innate immune system in patients with irritable bowel syndrome. *The American journal of gastroenterology* **104**, 404–10 (2009).
7. Kapel, N. et al. Fecal beta-defensin-2 in children with inflammatory bowel diseases. *Journal of pediatric gastroenterology and nutrition* **48**, 117–20 (2009).
8. Richter, M. et al. Influence of gestational age, cesarean section, and type of feeding on fecal human beta-defensin 2 and tumor necrosis factor-alpha. *Journal of pediatric gastroenterology and nutrition* **51**, 103–5 (2010).
9. Campeotto, F. et al. Fecal expression of human β-defensin-2 following birth. *Neo-natology* **98**, 365–9 (2010).
10. Shirin, T. et al. Antimicrobial peptides in the duodenum at the acute and convalescent stages in patients with diarrhea due to Vibrio cholerae O1 or enterotoxige-nic Escherichia coli infection. *Microbes and infection / Institut Pasteur* **13**, 1111–20 (2011).
11. Kabeerdoss, J. et al. Effect of yoghurt containing Bifidobacterium lactis Bb12® on faecal excretion of secretory immunoglobulin A and human beta-defensin 2 in healthy adult volunteers. *Nutrition journal* **10**, 138 (2011).
12. Savilahti, E. M. et al. Intestinal defensin secretion in infancy is associated with the emergence of sensitization and atopic dermatitis. *Clinical and experimental aller-gy* **42**, 405–11 (2012).
13. Lahtinen, S. J. et al. Probiotic cheese containing Lactobacillus rhamnosus HN001 and Lactobacillus acidophilus NCFM® modifies subpopulations of fecal lactobacilli and Clostridium difficile in the elderly. *Age* **34**, 133–43 (2012).
14. Kalach, N. et al. Intestinal permeability and fecal eosinophil-derived neurotoxin are the best diagnosis tools for digestive non-IgE-mediated cow's milk allergy in toddlers. *Clinical chemistry and laboratory medicine* **51**, 351–61 (2013).

Used symbols:

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