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Manual

β-Defensin 2 ELISA

For the in vitro determination of β-Defensin 2 in stool

Valid from 2020-05-01

REF **K 6500** Σ 96

REF **K 6500.20** Σ 20 x 96

+2 °C +8 °C

IVD

CE

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1. INTENDED USE

This assay is intended for the quantitative determination of β-defensin 2 in stool. For *in vitro* diagnostic use only.

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 α- or β-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. *E. coli*, *H. pylori* or *P. aeruginosa*).

A ß-defensin 2 deficiency can, for example, be observed in the intestinal mucous of patients 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 patients.

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.

Indications

- Reduced ß-defensin levels with Crohn's disease (HBD-2)
- Increased ß-defensin levels with Colitis Ulcerosa (HBD-2)

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity for cat. no.	
			K 6500	K 6500.20
K 6500	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
K 6500	CONJ	Conjugate concentrate, (goat anti-β-defensin 2, HRP-conjugated)	1 x 200 µl	15 x 200 µl
K 6500	STD	Standards, lyophilised	2 x 5 vials	25 x 5 vials

Cat. No.	Label	Kit components	Quantity for cat. no.	
			K 6500	K 6500.20
K 6500	STDBUF	Standard dilution buffer, ready-to-use	1 x 20 ml	5 x 20 ml
K 6500	CTRL1	Control, lyophilised, (see specification for range)	2 x 1 vial	25 vials
K 6500	CTRL2	Control, lyophilised (see specification for range)	2 x 1 vial	25 vials
K 6999.C.100	IDK Extract®	Extraction buffer concentrate *IDK Extract*® 2.5x	2 x 100 ml	10 x 100 ml
K 0002.15	SUB	Substrate (tetramethylbenzidine), 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*
- 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
- 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. 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.
- **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** 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 extraction buffer:** The **extraction buffer concentrate *IDK Extract*®** has to be diluted with ultrapure water **1:2.5** before use (100 ml *IDK Extract*® + 150 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 37 °C in a water bath. The ***IDK Extract*®** is stable at **2–8 °C** until the expiry date stated on the label. Extraction buffer (1:2.5 diluted *IDK Extract*®) can be stored in a closed flask at **2–8 °C for 4 months**.
- The **lyophilised standards (STD)** and **controls (CTRL)** are stable at **2–8 °C** until the expiry date stated on the label. Before use, the STD and CTRL have to be reconstituted with **500 µl of standard dilution buffer** (STDBUF) and mixed by gentle inversion to ensure complete reconstitution. Allow the vial content to dissolve for 10 minutes and then mix thoroughly. **Standards and controls** (reconstituted STD and CTRL) **can be stored at 2–8 °C for 2 weeks or at -20 °C for 4 weeks** and can be used **once after thawing.**
- **Preparation of the conjugate:** Before use, the **conjugate concentrate (CONJ)** has to be diluted **1:101** in **wash buffer** (100 µl CONJ + 10 ml wash buffer). 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

Raw stool

Raw stool ist stable for 2 days at room temperature (15–30 °C) , 7 days at 2–8 °C or at least 4 weeks at -20 °C.

Stool extract

Stool extract (1:100) is stable for 3 days at room temperature (15–30 °C), 7 days at 2–8 °C or 7 days at -20 °C. Avoid more than 2 freeze-thaw cycles.

Extraction of the stool samples

Extraction buffer (1:2.5 diluted *IDK Extract®*) 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 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 homogenisation using an applicator, inoculation loop or similar device.

b) Fill the **empty sample tube** with **1.5 ml extraction buffer** (1:2.5 diluted *IDK Extract®)* before using it with the sample. Important: Allow the extraction 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) Vortex 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

The supernatant of the sample preparation procedure (dilution I) is diluted **1:2 in wash buffer**. For example:

300 µl supernatant (dilution I) + **300 µl** wash buffer, mix well =**1:2** (**dilution II**)

This results in a final dilution of **1:200**.

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

7. ASSAY PROCEDURE

Principle of the test

This ELISA is designed for the quantitative determination of β-defensin 2 in stool.The β-defensin 2 in standards and samples is bound to an available excess of polyclonal antibodies against β-defensin 2, which are immobilised on the surface of the microtiter plate. After a washing step, to remove all interfering substances, the quantification of bound β-defensin 2 is carried out by adding a polyclonal anti β-defensin 2 antibody, which is horseradish peroxidase labelled. 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 proportional to the β-defensin 2 concentration in the sample. A dose response curve of the absorbance unit (optical density, OD at 450 nm) vs. concentration is generated, using the values obtained from the standard. β-defensin 2, 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.

We recommend to carry out the tests in duplicate.

1.	Wash the precoated microtiter plate **5 x with 250 µl ELISA wash buffer.** After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.
2.	Add each **100 µl standards/samples/controls** into the respective wells.
3.	Cover the strips and incubate for **1 hour** at room temperature (15–30 °C), **shaking* on a horizontal mixer.**
4.	Discard the contents of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.
5.	Add **100 µl conjugate** (diluted CONJ) in each well.
6.	Cover the strips and incubate for **1 hour** at room temperature (15–30 °C), **shaking* on a horizontal mixer**.
7.	Discard the contents of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.
8.	Add **100 µl substrate** (SUB) in each well.
9.	Incubate for **10–20 minutes**** at room temperature (15–30 °C) **in the dark.**
10.	Add **100 µl stop solution** (STOP) and mix well.
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.

* We recommend shaking the strips at 550 rpm with an orbit of 2 mm.

** 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.

Stool

The obtained results have to be multplied by the **dilution factor of 200** 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 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".

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

1 g stool is equivalent to 1 ml.

Stool (n = 101): 35 ng/ml

Based on Immundiagnostik AG 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 Immundiagnostik AG.

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

Accuracy – Precision

Repeatability (Intra-Assay); n = 30

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

Sample	Mean value [ng/ml]	CV [%]
1	17.70	4.2
2	81.44	3.0

Reproducibility (Inter-Assay); n = 24

The reproducibility was assessed with 4 stool samples under **varying** parameters (different operators, instruments, days and kit lots).

Sample	Mean value [ng/ml]	CV [%]
1	96.37	11.5
2	36.20	11.9
3	6.38	12.1
4	10.78	12.6

Accuracy – Trueness

The trueness states the closeness of the agreement between the result of a measurement and the true value of the measurand. Therefore, β-defensin 2 spikes with known concentrations were added to 8 different stool-samples. In the table below, 4 exemplary samples are shown:

Sample [ng/ml]	Spike [ng/ml]	Expected [ng/ml]	Obtained [ng/ml]	Recovery [%]
0.03	0.76	0.79	0.75	95.30
	0.10	0.13	0.14	110.00
	0.57	0.60	0.63	106.05
0.019	0.76	0.78	0.78	100.39
	0.10	0.12	0.14	113.45
	0.57	0.58	0.62	106.68
0.041	0.08	0.12	0.13	110.38
	0.17	0.21	0.21	99.04
	0.41	0.45	0.43	95.41
	0.93	0.98	0.87	89.26
0.027	0.08	0.11	0.11	99.63
	0.17	0.20	0.22	111.79
	0.41	0.43	0.43	98.71
	0.93	0.96	0.97	100.54

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 EP06-A with a serial dilution of 3 different stool samples.

For β-defensin 2 in stool, the method has been demonstrated to be linear from 0.065 to 2.200 ng/ml, showing a non-linear behaviour of less than ± 20 % in this interval.

Sample	Dilution	Expected [ng/ml]	Obtained [ng/ml]	Recovery [%]
A	1:200	0.873	0.873	100.00
	1:400	0.436	0.483	110.64
	1:800	0.218	0.241	110.48
	1:1600	0.109	0.121	110.98
	1:3200	0.055	0.065	118.60
B	1:200	1.866	1.866	100.00
	1:400	0.933	1.094	117.24
	1:800	0.466	0.528	113.27
	1:1600	0.233	0.253	108.52
	1:3200	0.117	0.123	105.81
	1:6400	0.058	0.069	118.57
C	1:200	2.200	2.200	100.00
	1:400	1.100	1.129	102.60
	1:800	0.550	0.520	94.48
	1:1600	0.275	0.262	95.12
	1:3200	0.138	0.139	101.14

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	0.0085 ng/ml
Limit of detection, LoD	0.0155 ng/ml
Limit of quantitation, LoQ	0.0232 ng/ml

The evaluation was performed according to the CLSI guideline EP-17-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 a range of compounds with structural similarity to β-defensin 2. There was no cross-reactivity observed.

Substance tested	Concentration added	Concentration obtained [ng/ml]	Conclusion
Lysozyme	30	< 0.0085	< LoB
Calprotectin	52	< 0.0085	< LoB
PMN elastase	10	< 0.0085	< LoB
Myeloperoxidase	100	< 0.0085	< LoB
EDN	16	< 0.0085	< LoB
α1-antitrypsin	10	< 0.0085	< LoB
Secretory IgA	600	< 0.0085	< 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 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 should 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 the 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 Extract*® 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. 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 amniotic 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. *Neonatology* **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 enterotoxigenic 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 allergy* **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

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