IMMUCHROM
IMMUCHROM GMBH

Manual

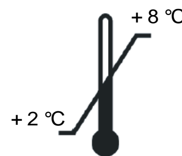
ß-Defensin 2

ELISA

For the determination of ß-defensin 2 in stool

Example Version

Valid from 28.12.2022



IC7200

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Example Version

1. Intended use

The *ImmuChrom* ELISA Kit is intended for the quantitative determination of ß-defensin 2 in stool. For in vitro diagnostic use by trained personnel in laboratories only.

2. Introduction

The group of ß-defensins are a class of antimicrobial cationic arginine-rich peptides and are part of the innate non-specific immune response. To date, nine different ß-defensins have been identified in humans. Defensins are produced by neutrophilic granulocytes and mucosal cells of the small and large intestine (5). This includes ß-defensin 2, which consists of 64 amino acids and has a molecular weight of 7 kDa.

Inflammation and microorganisms result in increased expression of ß-defensins (2). Defensin deficiency is observed in Crohn's disease patients. Due to the restricted barrier function of the intestinal mucosa, the inflammations typical of Crohn's disease can develop through bacterial invasion (3, 4). Defensin deficiency is discussed as a possible cause of the disease (6).

Defensins inhibit the activity of histamine-producing germ species (6). In addition, they have a modulating effect on the release of histamine from mast cells in the mucous membrane. At low histamine concentrations they cause release, at high concentrations they have an inhibitory effect (6). Defensin deficiency has an increased susceptibility to bacterial infections of the intestinal mucosa (6)

Taking non-steroidal anti-inflammatory drugs (e.g. aspirin, ibuprofen, diclofenac) or COX-2 inhibitors (e.g. celecoxib) can lead to enteropathies, which result in an increase in inflammatory parameters in the stool (8). Before carrying out the determination, the corresponding medication should therefore not be taken for a period of 14 days, so as not to influence the measurement of the degree of intestinal inflammation.

Example version

Applications

- inflammatory processes in the intestine
- integrity of the intestinal mucosa

The ImmuChrom complete ß-defensin 2 kit allows an easy, rapid and precise quantitative determination of ß-defensin 2 in biological samples. The kit includes all reagents ready to use for preparation of the samples.

3. General notes, warnings and precautions

This assay was produced and put on the market according to the IVD guidelines of 98/79/EC. All reagents of this kit are strictly intended for in vitro diagnostic use only.

Individual components from different batches and test kits should not be interchanged. The expiry dates stated on the relevant packaging must be observed.

The test kit reagents contain preservatives to protect against bacterial growth. Therefore contact with the skin and/or mucous membranes should be avoided.

The substrate TMB (tetramethylbenzidine) is toxic by ingestion and skin contact. In the event of contact with the skin, the affected area must be washed immediately with plenty of water and soap.

Avoid contact of the stop solution, which consists of acid, with the skin. It causes burns on contact. You should therefore work with protective gloves and goggles. In the event of contact, the burned area must be immediately and thoroughly rinsed with plenty of water. If necessary, a doctor should be consulted.

Adherence to the prescribed protocol for performing the test is essential. ImmuChrom GmbH assumes no liability for any damage caused by unauthorized changes in the test procedure.

The guidelines for carrying out quality control in medical laboratories must be observed. Appropriate controls must be carried along.

The reagents must not be used after the expiration date.

Wear disposable gloves when handling specimens or kit reagents and wash hands thoroughly afterwards. Do not pipette by mouth. Do not eat, drink, smoke, or put on makeup in areas where specimens or kit reagents are being handled.

Patient samples may contain unknown interfering substances. This can lead to false high or false low results.

The final clinical diagnosis should not be based on the results of a single test, but should be considered by a physician only after all clinical and laboratory results have been evaluated.

Example version

4. Material delivered in the test package

Artikel Nr.	Bezeichnung	Komponente	Menge
IC7200mtp	MTP	Microtiter plate coated	12 x 8 wells
IC7200wp	WASHBUF	ELISA wash buffer conc. 10 fold	100 ml
IC7400ex	EXT	Extraction buffer	150 ml
IC7200st	STD	Standards (1 ml) The concentrations are given in the specification	5 vials

IC7200ko	CTRL	Control 1 und 2 (1 ml) The concentrations are given in the specification	1 vial each
IC7200kg	CONJ	Conjugate, peroxidase labeled antibody	15 ml
IC7200vp	SAMPLEBUF	Sample buffer	20 ml
IC7200su	SUB	TMB substrate (tetramethylbenzidine)	15 ml
IC7200sp	STOPP	Stop solution	10 ml

5. Additional special equipment

- Centrifuge, 3000 x g
- Plastic reaction vials
- Stool sample extraction vials
- Vortex mixer
- Various pipettes
- Multichannel- or multipipette
- Foil to cover the microtiter plate
- Bidest. water
- ELISA reader with filter 450 nm (reference filter 620nm)
- Microtiter plate shaker

Example Version

6. Reagent preparation

Microtiter plate (MTP). Take the needed stripes and mount them on the holder. Please take care that the plate has reached room temperature (20–30 °C)before usage. Stripes which are not needed yet could be stored at 2-8 °C. Please dispose the holder when all stripes are used.

Wash buffer (WASHBUF). Dilute the wash buffer concentrate 1:10 with bidest. Water (1 part buffer + 9 parts aqua bidest.) The dilution is stable for 14 days at 2-8 °C.

Important: When storing the wash buffer concentrate at 2-8 °C crystallization could occur. Before dilution all crystals must be dissolved.

It is recommended to dilute only the amount of buffer which is used to process the given samples.

All other test reagents are stable at 2-8 °C up to the date of expiry stated on the label.

7. Specimen

Stool samples

The ß-defensin 2 is extracted by the extraction buffer out of the stool sample in a ratio of 1:100 (e.g. 10 mg/ml).

Extraction in stick vials

For the extraction stick vials could be used.

We recommend to mix **15 mg** stool with **1.5 ml** extraction buffer (EXT), then vortex it until the mixture is homogenous. Transfer the resulting slurry to a plastic vial and centrifuge it for 10 min at 3000 xg.

The supernatant is diluted 1:2 in sample buffer.

(75 µl supernatant + 75 µl sample buffer)

100 µl of the dilution are used in the test per well.

Example Version

8. Procedure

Principle of the method

The ß-defensin 2-ELISA test determines human ß-defensin 2 according to the "sandwich"-principle. ß-defensin 2 in sample, standard and controls binds to antibodies, which are coated to the microtiterplate. After a washing step a peroxidase labeled detection antibody is added. A second washing step is followed by the addition of the substrate which is converted to a colored product by the peroxidase. The reaction is terminated by the addition of an acidic stop solution. The optical densities are read at 450 nm (against the reference wavelength 620 nm) in a microtiterplate reader. The ß-defensin 2 concentration can be calculated from the standard curve.

Calibration: The test system is calibrated using a reference preparation of recombinant beta-defensin 2 purified from E. coli.

Sample preparation

All reagents and samples should have room temperature (20-30 °C) and should be mixed well before use.

The position of standards, controls and samples are noted on a protocol sheet.

1. Washing step

Take out the needed stripes of the microtiter plate and wash 1x with 250 µl diluted WASHBUF. Remove residual buffer by tapping the plate on absorbent paper after the washing step.

2. Incubation samples

Pipette **100 µl STD**, **CTRL** and diluted **samples** in double values in the microtiter plate.

The stripes are covered and incubated by shaking for **60 min** at room temperature (20-30 °C; 400 rpm, 2 mm orbit diameter).

3. Washing step

Discard the content of the microwells and wash 3x with 250 µl diluted WASHBUF. Remove residual buffer by tapping the plate on absorbent paper after the last washing step.

4. Incubation conjugate

Pipette **100 µl CONJ** in each microwell.

The stripes are covered and incubated by shaking for **60 min** at room temperature (20-30 °C; 400 rpm, 2 mm orbit diameter).

5. Washing step

Discard the content of the microwells and wash 3x with 250 µl diluted WASHBUF. Remove residual buffer by tapping the plate on absorbent paper after the last washing step.

6. Incubation substrate

Pipette **100 µl SUB** in each microwell.

Incubate for **10-15 min** under shaking at room temperature (20-30 °C; 400 rpm, 2 mm orbit diameter) in the dark.

7. Stopping reaction

Pipette **50 µl STOPP** in each microwell. Mix well.

8. Reading

Read the absorbance at 450 nm. If the microtiter plate reader allows to use a reference wavelength use 620nm as reference wavelength.

Reading should be done within 5 min after stopping reaction.

Example Version

9. Calculation of analytical results

For calculating the results we recommend to use the 4-parameter Marquart algorithm, if available.

Stool samples

Stool samples

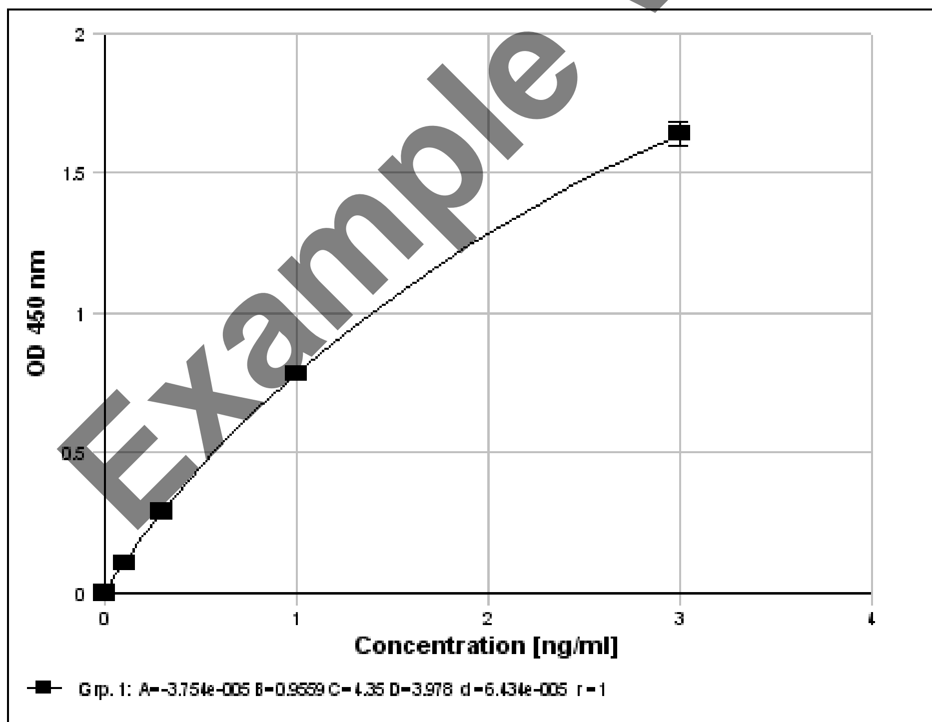
The obtained ß-defensin 2 concentration is multiplied with **200.**

Dilution 1: 15 mg in 1.5 ml corresponds to a factor **100** (assumption: 1 g stool = 1 ml)

Dilution 2: Factor **2**

Calculation: Conc. Patient [ng/ml] = obtained conc. [ng/ml] x 100 x 2

Standard curve



Example Version

The curve given above is only for demonstration. It must not be used for calculation of your samples

10 Internal quality control

Reference values

8 - 60 ng/ml stool

We recommend, that each laboratory should develop their own normal range. The values mentioned above are only for orientation and can deviate from other publicated data.

11. Validation data

Measuring range

The measuring range of the ß-Defensin 2 assay is between a sample concentration of 4 and 600 ng/ml.

Precision and reproducibility

Intra-Assay CV:	< 10 % (249.4 ng/ml)	[n = 10]
	< 10 % (68.60 ng/ml)	[n = 10]
	< 10 % (36.80 ng/ml)	[n = 10]
Inter-Assay CV:	< 15 % (117.5 ng/ml)	[n = 10]
	< 15 % (67.00 ng/ml)	[n = 10]
	< 15 % (36.70 ng/ml)	[n = 10]

Example Version

Detection limit

0.01 ng/ml

For the determination the zero-standard was measured 20 times. The 3-fold standard deviation was added to the mean value of the optical density. The respective concentration was read from the standard curve.

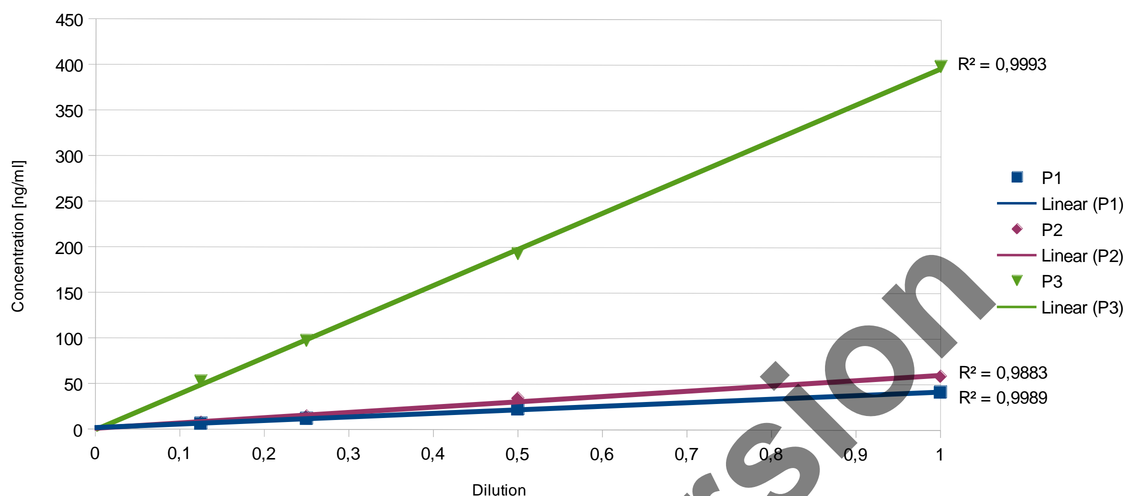
Limit of quantification

0.02 ng/ml

For the determination the zero-standard was measured 20 times. The 10-fold standard deviation was added to the mean value of the optical density. The respective concentration was read from the standard curve.

Linearity

The dilution of the samples are performed with sample buffer.



Sample	Dilution	Expected concentration [ng/ml]	Measured concentration [ng/ml]	Recovery [%]
P 1	-	42.40	42.40	-
	1:2	21.20	23.20	109.2
	1:4	10.60	12.40	116.7
	1:8	5.300	6.800	129.0
P 2	-	59.60	59.60	-
	1:2	29.80	34.90	117.3
	1:4	14.90	14.90	100.2
	1:8	7.500	7.900	106.2
P 3	-	399.3	399.3	-
	1:2	199.6	193.3	96.80
	1:4	99.80	97.70	97.80
	1:8	49.90	53.10	106.4

Example Version

Recovery

Sample	endogenous concentration [ng/ml]	Added concentration [ng/ml]	Expected concentration [ng/ml]	Measured concentration [ng/ml]	Recovery [%]
P 1	11.00	15.00	26.00	26.90	103.2
		45.00	56.00	59.80	106.7
		135.0	146.0	168.3	115.3
P 2	14.00	15.00	29.00	34.60	119.3
		45.00	59.00	70.50	119.4
		135.0	149.0	140.4	94.2
P 3	34.80	15.00	49.80	51.60	103.7
		45.00	79.80	71.00	88.9
		135.0	169.8	139.6	82.2

Cross reactivity

Cross reactivity to other ß-defensins like BD-1, BD-3 and BD-4 could not be detected in stool samples. The used concentration from the substances below was 100 ng/ml.

Example version

12. Limitations of the method

Stool samples with ß-defensin 2 concentrations above the standard curve should be diluted with sample buffer and measured again.

In case of diarrhea it is possible that even patients with an inflammation in the gut show normal values.

13. Disposal

The substrate (SUB) must be disposed as non-halogenated solvent. The stop solution (STOPP) could be neutralized with NaOH and if the pH value is neutral it can be disposed as salt solution. (**Important:** Reaction will produce heat, be careful)

Please refer to the appropriate national guidelines.

14. Literature references

1. Langhorst et al. (2009). Elevated Human β-Defensin-2 Levels Indicate an Activation of the Innate Immune System in Patients With Irritable Bowel Syndrome. *The American Journal of Gastroenterology* 104, Nr. 2: 404–10. doi:10.1038/ajg.2008.86.

2. Pero et al (2017). β-Defensins in the Fight against Helicobacter Pylori. *Molecules* 22, Nr. 3: 424. doi:10.3390/molecules22030424.

3. Wang et al (2007) Host–microbe Interaction: Mechanisms of Defensin Deficiency in Crohn's Disease. *Expert Review of Anti-Infective Therapy* 5, Nr. 6: 1049–57. doi:10.1586/14787210.5.6.1049.

4. Wehkamp et al. (2008). Barrier dysfunction due to distinct defensin deficiencies in small intestinal and colonic Crohn's disease. *Mucosal Immunology* 1, Nr. 1s: S67–74. doi:10.1038/mi.2008.48.

5. Siddiqui et al. (2017) *World J Gastrointest Pharmacol Ther* February 6; 8(1): 39-46

6. Kirkamm Hrsg. (2014) Spezielle Labordiagnostik in der naturheilkundlichen Praxis S. 13f. ISBN 978-3-437-56323-2

7. Kapel et al (2009) Fecal beta-defensin-2 in children with inflammatory bowel diseases. J Pediatr Gastroenterol Nutr. 2009 Jan;48(1):117-20. doi: 1097/MPG.0b013e318174e872.

8. Tibble JA, et al. 1999. High prevalence of NSAID enteropathy as shown by a simple faecal test. Gut. 45(3): 362-6

Example version