IMMUCHROM
IMMUCHROM GMBH

Manual

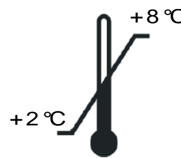
Calprotectin

ELISA

For the determination of calprotectin in stool

Example Version

Valid from 28.03.2024



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Example Version

1. Intended use

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

2. Introduction

Human calprotectin is a dimer which consists of the sub units S100A8 (10.835 kDa) and S100A9 (13.242 kDa). The monomers are able to bind calcium. The complex is located in the cytosol of neutrophils and is excreted to the intestine during inflammation (1). The concentration in faecal samples correlates with the severity of inflammatory processes in the intestine. The complex is resistant against enzymatic degradation. The measurement of Calprotectin represents an easy non-invasive analysis of intestinal inflammation with the possibility to differentiate between non-inflammatory irritable bowel disease and intestinal inflammation accompanied with morphological alteration of the intestinal mucosa (2,3).

In case of viral or bacterial infections of the gut the concentration of calprotectin in faeces is increased (4). Therefore, before further diagnostic clarification of a chronic inflammatory bowel disease and in case of an appropriate / corresponding clinic, the exclusion of an infectious cause (e.g. detection of pathogens, detection of pathogen-specific antibodies) is always necessary.

The use of non-steroidal anti-inflammatory drugs (e.g. aspirin, ibuprofen, diclofenac) or COX-2 inhibitors (e.g. celecoxib) can lead to enteropathies that lead to an increase in the calprotectin level in the stool (5,6). Before carrying out the determination, the corresponding medication should therefore not be taken for a period of 14 days if possible so as not to influence the measurement of the degree of intestinal inflammation (7). Administration of pantoprazole (20 mg/day) can lead to increased calprotectin levels. A measurement of calprotectin should be carried out 3 weeks after discontinuation of the medication (9).

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Applications

- inflammatory processes in the intestine
- clinical course of M. Crohn, colitis ulcerosa or after polyps removal
- safe differentiation between organic disorder of the intestine (chronic inflammatory bowel disease, infections, polyps, colon cancer) and functional disorder of the intestine (irritable bowel syndrome)

The ImmuChrom complete calprotectin kit allows an easy, rapid and precise quantitative determination of calprotectin 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.

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4. Material delivered in the test package

Article no.	Component	Description	Amount
IC7300mtp	MTP	Microtiterplate coated	12 x 8 wells
IC7300wp	WASHBUF	ELISA wash buffer conc. 10 fold	100 ml
IC7400ex	EXT	Extraction buffer	150 ml
IC7300st	STD	Standards (1.0 ml) The concentrations are given in the specification	5 vials

IC7300ko	CTRL	Control 1 und 2 (1.0 ml) The concentrations are given in the specification	1 vial each
IC7300kg	CONJ	Conjugate, peroxidase labeled antibody	15 ml
IC7310vp	SAMPLEBUF	Sample Buffer	120 ml
IC7300su	SUB	TMB substrate (tetramethylbenzidine)	15 ml
IC7300sp	STOPP	Stop solution	10 ml

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5. Additional special equipment

- Centrifuge, 3000 x g
- Eppendorf reaction vessels 1.5 ml
- Stool sample extraction vials
- Vortex mixer
- Various pipettes
- Multichannel- or multipipette
- Foil to cover the microtiter plate
- Bidest. Water
- Microtiter plate shaker
- ELISA reader with filter 450 nm (reference filter 620 nm)

6. Reagent preparation

Microtiter plate (MTP). Take the needed number of stripes and assemble them on the holder. Please take care that the plate has reached 20-30° C before usage. Stripes which are not needed yet must be stored at 2-8°C. Please do not dispose of the holder until all stripes are used.

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

Important: When storing the wash buffer concentrate at 2-8°C crystallization may 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, unless otherwise specified.

7. Specimen

Stool samples

Calprotectin is extracted by the extraction buffer out of the stool sample in a ration 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 1:38.5 in sample buffer (SAMPLEBUF). We recommend **20 µl** supernatant to mix with **750 µl** sample buffer. **100 µl** of the dilution are used in the test per well.

Please use only plastic vials and no glass vials.

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8. Procedure

Principle of the method

The calprotectin-ELISA test determines human calprotectin according to the "sandwich-principle". Calprotectin in sample, standard and controls binds to antibodies, which are coated to the microtiter plate. 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 microtiter plate reader. The calprotectin concentration can be calculated from the standard curve.

Calibration: The test system is calibrated from a reference preparation of recombinant and purified calprotectin from e. coli.

Sample preparation

All reagents and samples should 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 per cavity. 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.

   Cover the stripes with a cover film and incubate the microtiter plate by shaking for **60 min**(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 per cavity. 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.

   Cover the stripes with a cover film and incubate the microtiter plate by shaking for **60 min** (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 per cavity. 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 by shaking for **10-15 min** in the dark (20-30 °C; 400 rpm, 2 mm orbit diameter).

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 620 nm as reference wavelength.

   Reading should be done within 5 min after the stopping reaction.

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9. Calculation of analytical results

For calculating the results, we recommend to use the 4-parameter Marquardt algorithm.

Stool samples

The obtained calprotectin concentration is multiplied with **3.85.**

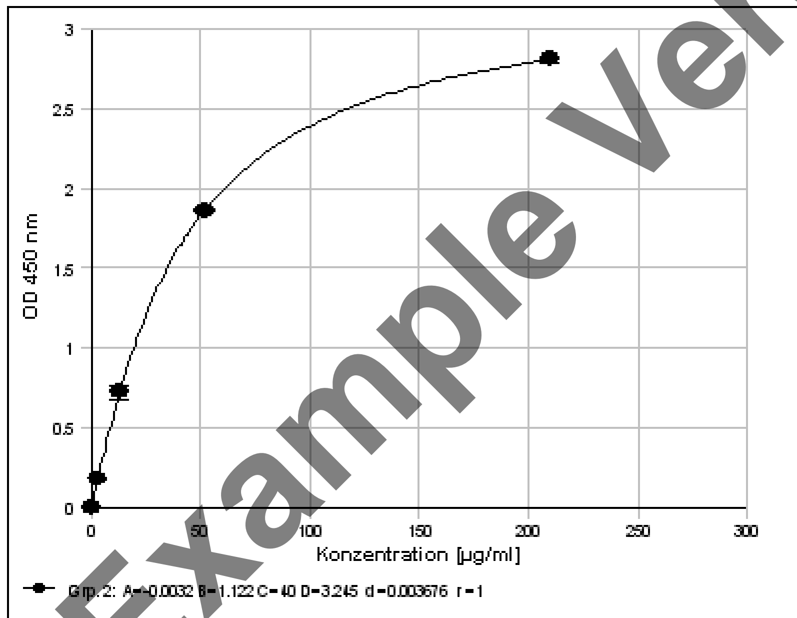
Dilution 1: 15 mg in 1.5 ml corresponds to a factor **100** (assumption: 1 g stool = 1 ml)

Dilution 2: Factor **38.5** (20 µl sample + 750 µl sample buffer)

Calculation: Conc. Patient [µg/ml] = obtained conc. [ng/ml] x 100 x 38.5/1000

Example Version

Standard curve



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

10. Internal quality control

Reference values

Stool: < 50 µg/ml

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

11. Validation data

Measuring Range

The measuring range of the calprotectin assays is between a sample concentration of 12.5 µg/ml and 800 µg/ml.

Precision and reproducibility

Intra-Assay CV:	4.8 % (20,4 µg/ml)	[n = 10]
	2.9 % (85,1 µg/ml)	[n = 10]
	7.4 % (166 µg/ml)	[n = 10]
Inter-Assay CV:	9.1 % (21,7 µg/ml)	[n = 10]
	7.8 % (94,2 µg/ml)	[n = 10]
	4.9 % (166 µg/ml)	[n = 10]

Detection limit

1.0 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

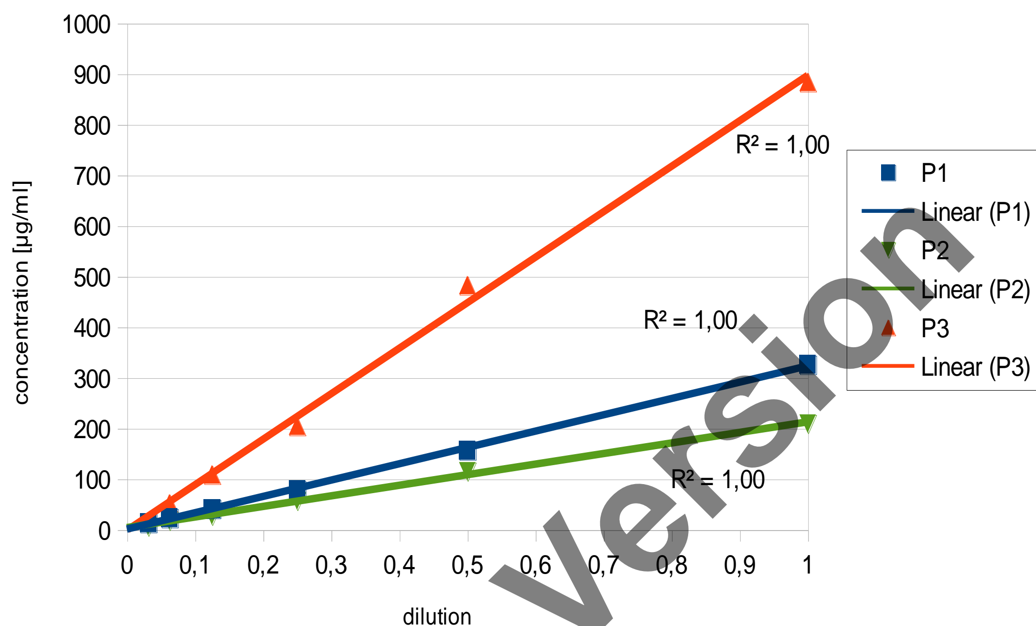
2.0 ng/ml

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.

Example Version

Linearity

The dilution of the samples was performed with sample buffer.



Example Version

Sample	Dilution	Expected concentration [µg/ml]	Measured concentration [µg/ml]	Recovery [%]
P 1	-		326	-
	1:2	163	157	96,32
	1:4	81,5	78,2	95,95
	1:8	40,8	41,4	101,6
	1:16	20,4	23,2	113,87
	1:32	10,2	13,4	131,35
P 2	-		209	-
	1:2	105	115	110,05
	1:4	52,25	57,2	109,47
	1:8	26,13	27,7	106,03
	1:16	13,06	17,7	135,5
	1:32	6,53	6,54	100,13
P 3	-		884	-
	1:2	442	483	109,28
	1:4	221	205	92,76
	1:8	111	109	98,64
	1:16	55,3	51,8	93,76

Recovery

Sample	endogenous concentration [μg/ml]	Added concentration [μg/ml]	Expected concentration [μg/ml]	Measured concentration [μg/ml]	Recovery [%]
P 1	21,05	48,8	69,8	61,5	88,11
		195	216	193	89,33
		780	801	754	94,13
P 2	18,24	48,8	67,0	65,2	97,33
		195	213	199	93,32
		780	798	822	102,98
P 3	23,88	48,8	72,6	66,3	91,28
		195	219	210	95,94
		780	804	802	99,77

Cross reactivity

Cross reactivity to other proteins could not be detected in stool samples.

12. Limitations of the method

Stool samples with calprotectin 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.

Example Version

14. Literature references

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8. Bressler B, et al. 2015. Clinicians´ guide to use of fecal calprotectin to identify and monitor disease activity in inflammatory bowel disease. Can J Gastroenterol Hepatol. 29(7): 369-72
9. Rendek, Z., Falk, M., Grodzinsky, E., Kechagias, S., Hjortswang, H., (2023), Oral omeprazole and diclofenac intake is associated with increased faecal calprotectin levels: a randomised open-label clinical trial, *European Journal of Gastroenterology and Hepathology*, 35(1), 52-58.

Example Version