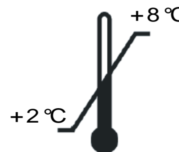
Manual

Lysozyme

ELISA

For the determination of lysozyme in stool

Valid from 10.06.2021



IC6900

ImmuChrom GmbH
Lise-Meitner-Str. 13
D 64646 Heppenheim
Tel.: ++49 6252 910084
Fax: ++ 49 6252 910070
Info@immuchrom.de
www.immuchrom.de

Table of contents Page

1. Intended use **3**

2. Introduction **3**

3. Warnings and precautions **3**

4. Material delivered in the test package **4**

5. Additional special equipment **5**

6. Reagent preparation **5**

7. Specimen **6**

Stool samples 6

8. Procedure **6**

Principle of the method 6

Sample preparation 7

9. Calculation of analytical results **8**

Standard curve 8

10. Internal quality control **9**

Reference values 9

11. Validation data **9**

Precision and reproducibility 9

Linearity 9

Detection limit 9

Recovery 10

Cross reactivity 10

12. Limitations of the method **10**

13. Disposal **10**

14. Literature references **11**

15. General notes Fehler! Textmarke nicht definiert.

1. Intended use

The *ImmuChrom* ELISA Kit is intended for the quantitative determination of lysozyme in stool. For *research* use only.

2. Introduction

Lysozyme is a relatively small enzyme consisting of a 129 amino acid polypeptide chain with a molecular weight of 14.6 kDa.

Lysozyme is a glycosidase that, as a component of the innate immune system, is primarily directed against the murein-containing cell wall of gram-positive bacteria. Therefore, it is also called muramidase because it can cleave the murein (peptidoglycan) from bacterial walls by hydrosylating the beta-1,4-glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid.

Lysozyme is found in many secretions of the human body, including tear fluid, saliva, blood serum and cerebrospinal fluid. It is produced in the tissues of the respiratory tract, kidneys, and intestinal mucosa, as well as by neutrophil granulocytes and macrophages. [2;3]

Under physiological conditions, about 80% of the lysozyme in blood plasma is due to neutrophil granulocyte degradation. [4]

Lysozyme is normally undetectable or detectable in small amounts in the intestinal contents. However, fecal lysozyme may occur through intestinal granulocytes. Lysozyme can be detected in all cells of the inflammatory infiltrate in Crohn's disease. Furthermore, lysozyme can be actively secreted into the intestinal lumen by monocytes and macrophages. [1;]

Applications

- Inflammatory processes in the intestine
- Detection of a disturbed immunological barrier at the intestinal mucosa

3. Warnings and precautions

All reagents of this kit are strictly intended for *research* use only.

Do not interchange kit components from different lots.

The stop solution (STOP) contains acid and has to be handled carefully. It is corrosive and causes burns. It should be handled with gloves, eye protection and appropriate protective clothing in a hood. Any spill should be wiped up immediately with copious quantities of water. Do not breath vapor and avoid inhalation. In case of an accident or indisposition contact a physician immediately.

The substrate TMB (tetramethyl benzidine) is toxic by ingestion and contact with the skin. Any spill should be wiped out immediately with copious quantities of water.

Wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.

Do not pipette by mouth.

Do not eat, drink, smoke or apply makeup in areas where specimens or kit reagents are handled.

The reagents of the test kit contain bactericides to protect against bacterial growth. Avoid the contact with the skin or mucous membrane.

Reagents should not be used beyond the expiration date shown on kit label.

Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. During handling of all kit reagents, controls and serum samples observe the existing legal regulations.

4. Material delivered in the test package

Article no.	Component	Description	Amount
IC6900mtp	MTP	Microtiter plate coated	12 x 8 wells
IC6900wp	WASHBUF	Lysozyme ELISA wash buffer conc. 10xfold	100 ml
IC6900vp	SAMPLEBUF	Sample buffer	20 ml
IC6900st	STD	Standards (1 ml) (0; 0.55; 1.65; 5.0 ng/ml)	4 vials
IC6900ko	CTRL	Control 1 and 2 (1 ml)	1 vial each
IC6900kg	CONJ	Conjugate, peroxidase-labeled antibody	15 ml
IC6900su	SUB	TMB substrate (tetramethylbenzidine)	15 ml
IC6900sp	STOPP	Stop solution	10 ml

5. Additional special equipment

- Centrifuge, 3000 xg
- Plastic vials
- 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 room temperature 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.

<u>Important:</u> 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

The lysozyme is extracted by the diluted wash buffer out of the stool sample.

Extraction in Stool extraction vials

In a stool sample extraction vial mix 15 mg stool with 0.75 ml diluted wash buffer, then vortex it until the mixture is homogenous. Transfer the resulting slurry to a plastic vial and centrifuge it for 10 min at 3000xg.

The supernatant is diluted 1:10 in SAMPLEBUF. We recommend 20 µl supernatant to mix with 180 µl SAMPLEBUF. 100 µl of the dilution are used in the test per well.

Please use only plastic vials and no glass vials.

8. Procedure

Principle of the method

The lysozyme-ELISA test determines human lysozyme according to the “sandwich”-principle. Lysozyme 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 lysozyme concentration can be calculated from the standard curve.

Sample preparation

All reagents and samples should be prewarmed to 20°C – 30°C and mixed well before use.

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

1. **Washing step**

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

2. **Samples incubation**

   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).

3. **Washing step**

   Discard the content of the microwells and wash 5x with 250 µl diluted WASHBUF. Remove residual buffer by tapping the plate on absorbent paper after the last washing step.

4. **Conjugate incubation**

   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).

5. **Washing step**

   Discard the content of the microwells and wash 5x with 250 µl diluted WASHBUF. Remove residual buffer by tapping the plate on absorbent paper after the last washing step.

6. **Substrate incubation**

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

   Incubate for **10 - 15 min** in the dark (20-30 °C)

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 stopping reaction.

9. Calculation of analytical results

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

Stool samples

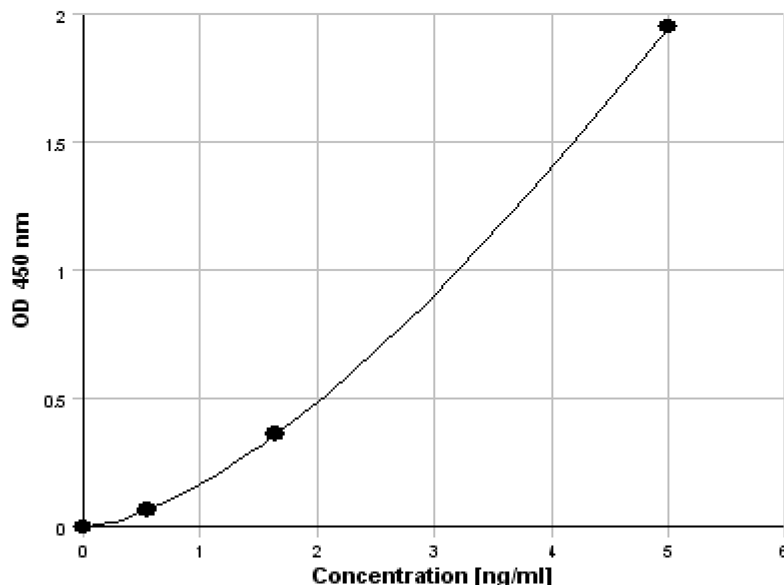
The obtained lysozyme concentration is multiplied with **0,5**

Dilution 1: 15 mg in 0.75 ml corresponds to a factor **50** (assumption: 1 g stool = 1 ml)

Dilution 2: Factor **10** (20 µl sample + 180 ml sample buffer)

Calculation: Conc. $_{\text{Patient}}$ [µg/ml] = obtained conc. [ng/ml] x 50 x 10 / 1000

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: < 600 ng/g 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 published data.

11. Validation data

Precision and reproducibility

Intra-Assay CV:	8.6 % (69.5 ng/ml)	[n = 10]
	3.4 % (13.5 ng/ml)	[n = 10]
	4.0 % (2.3 ng/ml)	[n = 10]
Inter-Assay CV:	5.1 % (18.4 ng/ml)	[n = 10]
	6.1 % (4.8 ng/ml)	[n = 10]
	8.0 % (1.6 ng/ml)	[n = 10]

Linearity

The linearity of the test ranges from 150 to 2500 ng/g stool.

The dilution of the samples was performed with sample buffer.

Detection limit

Stool 150 ng/g

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.

Recovery

The mean value of the recovery 92,4%.

Cross reactivity

Cross-reactivities to other proteins, especially to lysozyme from chicken egg white, were not found.

12. Limitations of the method

Stool samples with lysozyme concentrations above the standard curve should be diluted with sample buffer and measured again.

In case of strong 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) can be neutralized with NaOH and if the pH value is neutral it can be disposed as salt solution. (**important:** this reaction produces heat and should be handled carefully).

Please refer to the appropriate national guidelines.

14. Literature references

1 - Johan Brouwer, Trudi van Leeuwen-Herberts und Marjo Otting-van de Ruit: Determination of lysozyme in serum, urine, cerebrospinal fluid and feces by enzyme immunoassay. In: Clinica Chimica Acta. 142, Nr.1, 15.September 1984, S.21-30.

2 - Lien Callewaert, Chris W. Michiels: Lysozymes in the animal kingdom. In: Journal of Biosciences. 35, Nr.1, März 2010, S.127–160.

3 - H. A. McKenzie und F.H. White (Jr.): Lysozyme and α-lactalbumin: structure, function and interrelationships. In: Advances in Protein Chemistry. 41, 1991, S.173-315.

4 - J. P. van de Merwe, J. Lindemans und G. J. Mol: Plasma lysozyme levels and decay of neutrophilic granulocytes in patients with Crohn's disease. In: Hepatogastroenterology. 27, Nr.2, April 1980, S.130-134.