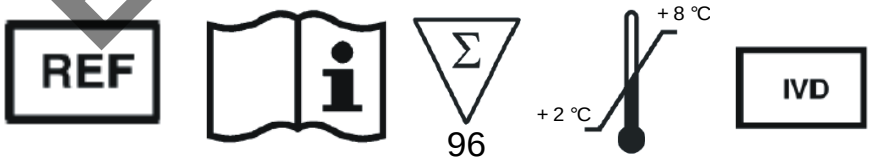
Manual

Lysozyme

ELISA

For the determination of lysozyme in stool

Valid from 28.04.2023



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1. Intended use

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

2. Introduction

Lysozyme is a relatively small enzyme consisting of a polypeptide chain of 129 amino acids and has a molecular weight of 14.6 kDa.

Lysozyme is a glycosidase which, as a component of the innate immune system, is primarily directed against the murein-containing cell wall of gram-positive bacteria. Hence 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 tears, saliva, blood serum and cerebrospinal fluid. It is produced in the tissues of the respiratory tract, kidneys and intestinal mucosa, as well as by neutrophils and macrophages. (2;3)

Under physiological conditions, about 80% of the lysozyme in the blood plasma is due to the breakdown of neutrophilic granulocytes. (4)

Normally, lysozyme is not detectable in the intestinal contents, or only in small amounts. However, fecal lysozyme can occur due to 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;5)

Taking non-steroidal anti-inflammatory drugs (e.g. aspirin, ibuprofen, diclofenac) or COX-2 inhibitors (e.g. celecoxib) can cause irritation and damage to the lining of the gastrointestinal tract, ranging from small superficial lesions (erosions) to deeper ulcers (ulcers) to lighter and heavy bleeding. Therefore, if possible, the relevant medication should not be taken for a period of 14 days prior to carrying out the determination, in order not to influence the measurement of the degree of intestinal inflammation (10).

Applications

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

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 test kit contains components of human origin. The starting reagents were tested for antibodies against HIV1/2, hepatitis B and anti-HCV using immunoassay methods. All parameters tested were found negative. As a precautionary measure, all test kit reagents should always be treated as potentially infectious material in accordance with health care accident prevention regulations.

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 wear 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.

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 each) (0; 0.55; 1.65; 5.0 ng/ml)	4 vials
IC6900ko	CTRL	Control 1 and 2 (1 ml each)	2 vials
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, 3000xg
- Plastic reaction vials
- Stool sample extraction vials
- Vortex mixer
- Various pipettes
- Multichannel- or multipipette
- Foil to cover the microtiterplate
- Bidest. water
- Microtiter plate shaker
- ELISA reader with filter 450 nm (reference filter 620)

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

Lysozyme is extracted by the diluted wash buffer out of the stool sample in a ratio of 1:50 (e.g. 20 mg/ml).

Extraction in Stool extraction vials

For the extraction stick vials could be used.

We recommend to 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 3000 x g.

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, 400 rpm, 2 mm orbit diameter).

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, 400 rpm; 2 mm orbit diameter).

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, 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 stopping reaction.

9. Calculation of analytical results

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

Stool samples

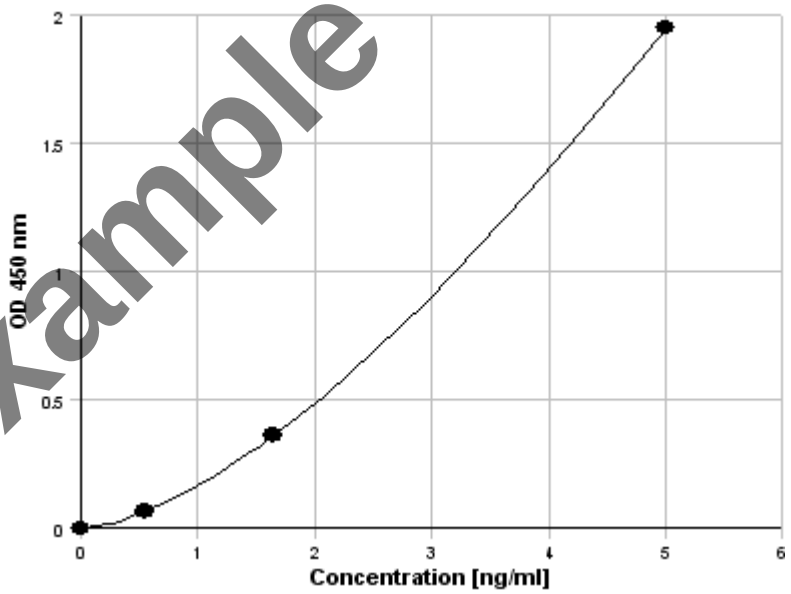
The obtained lysozyme concentration is multiplied with **0,5** to get patient concentration as µg/ml

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

Measuring range

The measuring range of lysozyme is between a sample concentration of 0.55 - 5 ng/ml (is equivalent to a sample concentration of 275 – 2500 ng/ml).

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]

Detection limit

0.3 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.

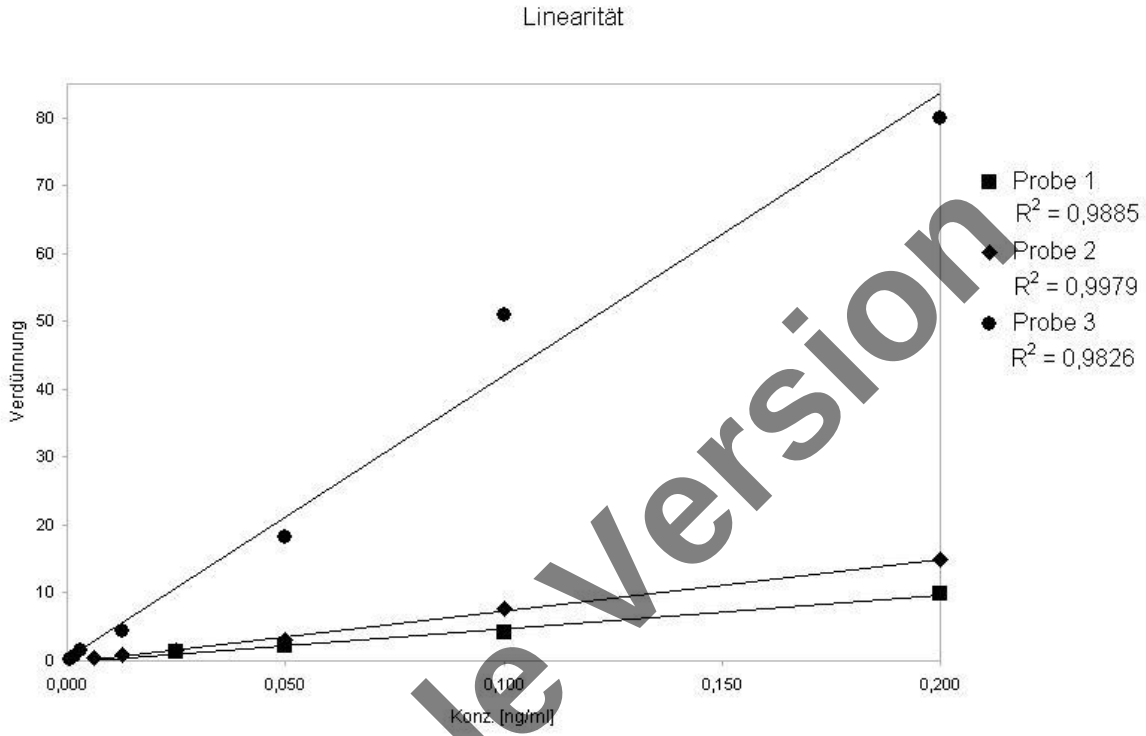
Limit of quantification

0.5 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 was done with sample buffer.



Sample	Dilution factor	Expected [ng/ml]	Measured [ng/ml]	Recovery [%]
1	-- 1:2 1:4 1:8	-- 4.99 2.49 1.25	9.97 4.17 2.28 1.36	-- 83.6 91.4 109.2
2	-- 1:2 1:4 1:8 1:16 1:32	-- 7.45 3.73 1.86 0.93 0.47	14.91 7.67 3.18 1.52 0.90 0.44	-- 102.9 85.3 81.6 96.1 95.1
3	-- 1:2 1:4 1:16 1:64 1:128 1:256	-- 40.03 20.01 5.00 1.25 0.63 0.31	80.06 50.87 18.16 4.35 1.49 0.67 0.3	-- 127.1 90.6 87.0 119.4 106.4 97.1

Recovery

Sample	Endogenous [ng/ml]	Added	Expected [ng/ml]	Meassured [ng/ml]	Recovery [%]
1	0.89	54.07 12.61 2.69	54.97 13.51 3.58	41.57 11.79 2.79	76.88 93.48 103.85
2	0.57	11.07 2.18 0.76	11.64 2.75 1.32	12.2 2.5 1.1	104.83 91.01 83.23

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

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