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Manual

Lysozyme ELISA

For the in vitro determination of lysozyme in stool

Valid from 2020-05-01

REF **K 6900**

Σ 96

+2°C +8°C

IVD

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1. INTENDED USE

This Immundiagnostik AG assay is an enzyme immunoassay intended for the quantitative determination of lysozyme in stool. For *in vitro* diagnostic use only.

2. INTRODUCTION

Lysozyme (muramidase) is a protein with a molecular weight of approx. 15 kDa and belongs to the group of alkaline glycosidases. Lysozyme is produced by granulocytes, monocytes and macrophages. The main source for faecal lysozyme are the intestinal granulocytes. Lysozyme can be detected in all cells of the inflammatory infiltrate during an acute attack of Crohn's disease. To some extent, lysozyme is also secreted actively by mononuclear cells into the bowel lumen.

Indications

- Diagnosis and monitoring of Crohn's Disease
- Early diagnosis of rejection reactions in kidney transplantation cases
- Differential diagnosis and monitoring of leukosis
- Diagnosis and treatment monitoring of urinary tract infections in children
- Differential diagnosis between viral and bacterial meningitis in children
- Identification of sepsis in neonates

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity
K 6900	PLATE	Microtiter plate, pre-coated	12 x 8 wells
K 0001.C.100	WASHBUF	Wash buffer concentrate, 10 x	2 x 100 ml
K 6999.C.100	IDK Extract®	Extraction buffer concentrate *IDK Extract®* 2.5x	1 x 100 ml
K 6900	CONJBUF	Conjugate dilution buffer, ready-to-use	2 x 15 ml
K 6900	CONJ	Conjugate concentrate (rabbit-anti-lysozyme, peroxidase-labelled)	1 x 50 µl
K 6900	STD	Calibrators, ready-to-use (0; 1.1; 3.3; 10; 30 ng/ml)	5 x 1 ml
K 6900	CTRL1	Control, ready-to-use (see specification for range)	1 x 1 ml

Cat. No.	Label	Kit components	Quantity
K 6900	CTRL2	Control, ready-to-use (see specification for range)	1 x 1 ml
K 0002.15	SUB	Substrate (tetramethylbenzidine), ready-to-use	1 x 15 ml
K 0003.15	STOP	Stop solution, ready-to-use	1 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 **for 4 months** at **2–8 °C**.
- **Preparation of the conjugate:** Before use, the **conjugate concentrate (CONJ)** has to be diluted **1:1001** in **conjugate dilution buffer (CONJBUF)** (10 µl CONJ + 10 ml CONJBUF). The CONJ is stable at **2–8 °C** until the expiry date stated on the label. **Conjugate** (1:1001 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 storage

The sample stability is as follows:

Raw stool: 3 days at room temperature (15–30 °C) and 2–8 °C as well as 3 months at -20 °C.

Stool extracts (1:100): 1 day at room temperature (15–30 °C), 5 days at 2–8 °C or 7 days at -20 °C, maximum 1 freeze-thaw cycle.

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 sample 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 stool sample tube** with 1.5 ml **sample extraction buffer** (1:2.5 diluted *IDK Extract®)* before using it with the sample. **Important:** Allow the sample extraction buffer to reach room temperature.

c) Unscrew the tube (yellow part of cap) to open. Insert the orange 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 sample extraction 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:

125 µl supernatant (dilution I) + **125 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

The assay utilises the "sandwich" technique with two selected antibodies that recognise human lysozyme.

Standards, controls and diluted samples, which are assayed for human lysozyme, are added into the wells of a micro plate coated with a high affine anti-human lysozyme antibody. During the first incubation step, lysozyme is bound by the immobilised antibody. Then a peroxidase-conjugated anti-human lysozyme antibody is added into each microtiter well and a "sandwich" of capture antibody - human lysozyme – peroxidase-conjugate is formed. Tetramethylbenzidine is used as peroxidase substrate. Finally, an acidic stop solution is added to terminate the enzymatic reaction. The colour changes from blue to yellow. The intensity of the yellow colour is directly proportional to the concentration of lysozyme. A dose response curve of the absorbance unit (optical density, OD at 450 nm) vs. concentration is generated using the values obtained from the standards. Lysozyme present in the 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 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.

For automated ELISA processors, the given protocol may need to be adjusted according to the specific features of the respective automated platform. For further details please contact your supplier or Immundiagnostik AG.

We recommend to carry out the tests in duplicate.

1.	**Before use**, wash the wells **5 times** with **250 µl wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
2.	Add **100 µl standards/controls/ prepared samples** into the respective wells.
3.	Cover the strips and incubate for **1 hour** at room temperature (15-30 °C) **on a horizontal shaker***.

4.	Discard the contents of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
5.	Add **100 µl conjugate** (diluted CONJ) into each well.
6.	Cover the strips and incubate for **1 hour** at room temperature (15-30 °C) **on a horizontal shaker***.
7.	Discard the contents of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
8.	Add **100 µl substrate** (SUB) into each well.
9.	Incubate for **10–20 min**** at room temperature (15-30 °C) **in the dark**.
10.	Add **100 µl stop solution** (STOP) into each well 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 multiplied 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:

Analytical sensitivity × *sample dilution factor to be used*

Analytical sensitivity 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

Based on Immundiagnostik AG studies of stool samples of apparently healthy persons (n = 80), a reference value of lysozyme < 600 ng/ml was estimated.

We recommend each laboratory to establish its own reference range.

11. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

Intra-Assay (n = 18)

Sample	Lysozyme [ng/ml]	CV [%]
A	6.7	7
B	1.4	10

Inter-Assay (n = 16)

Sample	Lysozyme [ng/ml]	CV [%]
A	5.9	14
B	1.7	12

Spiking Recovery

Two samples were spiked with different lysozyme concentrations and measured using this assay (n = 2).

Sample	Unspiked Sample [ng/ml]	Spike [ng/ml]	Lysozyme expected [ng/ml]	Lysozyme measured [ng/ml]
A	1.5	3	4.5	5.5
		6	7.5	7.1
B	0.75	0.7	1.4	1.5
		1.7	2.4	2.7
		5	5.8	5.7

Analytical Sensitivity

The zero standard was measured 20 times. The detection limit was set as B_0 + 2 SD and estimated to be 0.5 ng/ml.

Specificity

No cross reactivity to other plasma proteins was observed:

- α1-Antitrypsin < 0.1 %
- Albumin < 0.1 %
- PMN elastase < 0.1 %
- sIgA < 0.1 %
- Haemoglobin < 0.1 %
- Hb/Hp complex < 0.1 %
- CRP < 0.1 %
- Pancreatic amylase < 0.1 %
- Chymotrypsin < 0.1 %
- IgE < 0.1 %

Dilution recovery

Two patient samples were serially diluted and analysed. The results are shown below (n = 2):

Sample	Dilution	Lysozyme expected [ng/ml]	Lysozyme measured [ng/ml]
A	undiluted	15.3	15.3
	1:2	7.7	7.5
	1:4	3.8	3.9
	1:8	1.9	< detection limit
B	undiluted	9.3	9.3
	1:2	4.65	6.0
	1:4	2.3	4.1
	1:8	1.16	1.6

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 must 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. REFERENCES

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4. Volman, Julia J, Ronald P Mensink, Wim a Buurman, and Jogchum Plat. 2011. "In Vivo Effects of Dietary (1→3), (1→4)-B-D-Glucans from Oat on Mucosal Immune Responses in Man and Mice." *Scandinavian Journal of Gastroenterology* **46** (5) (May): 603–10. doi:10.3109/00365521.2010.545830.
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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