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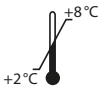
Manual

Lactoferrin ELISA

For the in vitro determination of lactoferrin in stool

Valid from 2019-12-05

REF **K 6870**



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1. INTENDED USE

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

2. INTRODUCTION

Lactoferrin is a 76 kDa iron-binding glycoprotein which is synthesised and stored in the secondary granules of neutrophils. It is also present in several secretory fluids, such as milk, saliva, tears, and nasal secretions.

Lactoferrin can exist in different polymeric forms ranging from monomers to tetramers; it tends to polymerise especially at high concentrations.

The physiological activities of lactoferrin include regulation of iron homeostasis, innate defense against a broad range of microbial infections, anti-inflammatory activity, regulation of cellular growth and differentiation and protection against cancer development and metastasis.

During intestinal inflammation, polymorphonuclear neutrophils infiltrate the mucosa and release lactoferrin by degranulation, which results in an increased excretion of lactoferrin into the faeces. Faecal lactoferrin is therefore a marker for neutrophilic intestinal inflammation.

Indications

- Detection of intestinal inflammatory activity
- Monitoring of disease activity in inflammatory bowel disease (IBD)
- Prediction of relapse of IBD
- Assessment of IBD treatment response

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity
K 6870	PLATE	Microtiter plate, pre-coated	12 x 8 wells
K 0001.C.100	WASHBUF	Wash buffer concentrate, 10x	2 x 100 ml
K 6999.C.100	IDK Extract®	Extraction buffer concentrate *IDK Extract®*, 2,5x	1 x 100 ml
K 6870	CONJ	Conjugate concentrate (rabbit anti human lactoferrin)	1 x 200 µl

Cat. No.	Label	Kit components	Quantity
K 6870	STD	Standards, lyophilised (see specification for concentrations)	4 x 5 vials
K 6870	CTRL1	Control, lyophilised (see specification for range)	4 x 1 vial
K 6870	CTRL2	Control, lyophilised (see specification for range)	4 x 1 vial
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
K 6870	SAMPLEBUF	Sample dilution buffer, ready-to-use	1 x 50 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 dispenser or repeating dispenser
- Vortex mixer
- 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. PREPARATION AND STORAGE 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 at **2–8 °C for 4 months**.
- The **lyophilised standards (STD)** and **controls (CTRL)** are stable at **2–8 °C** until the expiry date stated on the label. **Reconstitution** details are given in the **specification data sheet**. **Standards and controls** (reconstituted STD and CTRL) **can be stored at 2–8 °C or -20 °C for 7 days.**
- **Preparation of the conjugate:** Before use, the **conjugate concentrate (CONJ)** has to be diluted **1:101** in **wash buffer** (100 µl CONJ + 10 ml wash buffer). The CONJ is stable at **2–8 °C** until the expiry date stated on the label. **Conjugate** (1:101 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 stability and storage

Raw stool

Raw stool is stable for 3 days at room temperature (15-30 °C), 4 days at 2-8 °C or up to 6 month at -20 °C.

Stool extract

Stool extract is stable for 9 days at room temperature (15-30 °C), at 2-8 °C or at -20 °C. Avoid more than three freeze-thaw cycles.

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 yellow 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 **1:10 in sample dilution buffer**. For example:

- **50 µl** supernatant (dilution I) + **450 µl** sample dilution buffer, mix well = **1:10** (**dilution II**). This results in a final dilution of 1:1 000.

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

7. ASSAY PROCEDURE

Principle of the test

This ELISA is intended for the quantitative determination of lactoferrin in stool. In a first incubation step, the lactoferrin in the samples is bound to polyclonal antibodies, immobilised to the surface of the microtiter wells. To remove all unbound substances, a washing step is carried out. In a second incubation step, a peroxidase-labelled conjugate (rabbit anti human lactoferrin) is added which recognises specifically the bound lactoferrin. After another washing step to remove all unbound substances, the solid phase is incubated with the substrate, tetramethyl-benzidine (TMB), which reacts with the peroxidase. An acidic stop solution is added to stop the reaction. The colour changes from blue to yellow. The intensity of the yellow colour is directly proportional to the concentration of lactoferrin. 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. Lactoferrin, present in the patient 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.

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.

Take as many microtiter strips as needed from the 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.

We recommend to pipet the standards and controls in duplicate.

1.	Add each **100 µl standards/controls/diluted samples** into the respective wells.
2.	Cover the strips and incubate for **30 min** at room temperature (15–30 °C).
3.	Discard the content 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.
4.	Add **100 µl conjugate** in each well.
5.	Cover the strips and incubate for **30 min** at room temperature (15–30 °C).
6.	Discard the content 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.
7.	Add **100 µl substrate** (SUB) into each well.
8.	Incubate for **10–20 min*** at room temperature (15–30 °C) in the **dark**.
9.	Add **100 µl stop solution** (STOP) into each well and mix well.
10.	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.

* 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 calibrator 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 pairs of values should be examined before the automatic evaluation of the results. If this option is not available with the used program, a control of the paired values should be done manually.

Stool samples

The obtained results have to be multplied with the **dilution factor of 1 000** to get the actual concentrations.

In case **another dilution factor** has been used, multiply the obtained result with 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:

LoB × *sample dilution factor to be used*

LoB 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

We recommend each laboratory to establish its own reference concentration range.

Reference values in stool

1 g stool is equivalent to 1 ml.

Normal value: < 7,2 µg/ml

11. PERFORMANCE CHARACTERISTICS

Accuracy – Precision

Repeatability (Intra-Assay); n = 40

The repeatability was assessed with 2 stool samples under **constant** parameters (same operator, measurement system, day and kit lot).

Sample	Mean value [µg/ml]	CV [%]
1	28.75	4.1
2	6.56	4.8

Reproducibility (Inter-Assay); n = 16

The reproducibility was assessed with 2 stool samples under **varying** parameters (different operators, measurement systems, days and kit lots).

Sample	Mean value [µg/ml]	CV [%]
1	17.66	9.5
2	4.45	14.0

Accuracy – Trueness

The trueness states the closeness of the agreement between the result of a measurement and the true value of the measurand. Therefore, lactoferrin spikes with known concentrations were added to 6 different stool samples. The results below were obtained without consideration of the sample dilution factor.

Sample [ng/ml]	Spike [ng/ml]	Expected [ng/ml]	Obtained [ng/ml]	Recovery [%]
1.27	5.7	6.94	7.43	107.03
	28.4	29.61	29.70	100.30
	56.8	58.06	54.10	93.17
	113.6	114.85	98.37	85.66
16.54	5.7	22.06	20.88	94.66
	28.4	44.12	43.13	97.76
	56.8	73.18	63.25	86.43
	113.6	129.81	105.10	80.96
123.88	5.7	128.32	124.44	96.97
	28.4	146.09	135.13	92.50
	56.8	179.44	155.68	86.76
	113.6	235.00	206.39	87.82
2.23	24.4	26.09	23.30	89.30
	32.6	34.05	33.63	98.75
	48.9	49.97	52.71	105.49
	65.1	67.36	76.21	113.12
5.36	24.4	28.44	24.78	87.13
	32.6	36.15	34.25	94.75
	48.9	51.53	51.94	100.78
	65.1	66.92	71.72	107.17
14.66	24.4	35.41	29.89	84.42
	32.6	42.35	39.88	94.16
	48.9	56.18	55.26	98.36
	65.1	70.02	75.26	107.48

Linearity

The linearity states the ability of a method to provide results proportional to the concentration of analyte in the test sample within a given range. This was assessed according to CLSI guideline EP6-A with a serial dilution of 2 different stool samples.

For lactoferrin in stool, the method has been demonstrated to be linear from 11.62 to 151.46 ng/ml based on the standard curve without considering possibly used sample dilution factors, showing a non-linear behaviour of less than ± 20 % in this interval.

Sample	Dilution	Expected [ng/ml]	Obtained [ng/ml]	Recovery [%]
A	1:250	97.71	97.71	100.00
	1:375	65.14	78.20	120.06
	1:500	48.85	58.59	119.94
	1:750	32.57	35.65	109.45
	1:1 000	24.43	27.70	113.40
B	1:500	151.46	151.46	100.00
	1:1 000	75.73	83.36	110.07
	1:2 000	37.87	45.62	120.47
	1:2 500	30.29	33.85	111.73
	1:4 000	18.93	22.60	119.37
	1:8 000	9.47	11.62	122.77

Analytical sensitivity

The following values have been estimated based on the concentrations of the standards without considering possibly used sample dilution factors.

Limit of blank, LoB	0.222 ng/ml
Limit of detection, LoD	0.416 ng/ml
Limit of quantitation, LoQ	0.416 ng/ml

The evaluation was performed according to the CLSI guideline EP17-A2. The specified accuracy goal for the LoQ was 20 % CV.

Analytical specificity

The specificity of the antibody was tested by measuring the cross-reactivity against a range of compounds with structural similarity to lactoferrin. There was no cross-reactivity observed.

Substance tested	Concentration added [ng/ml]	Concentration obtained [ng/ml]	Conclusion
PMN elastase	1 000	< 0,222	< LoB
Calprotectin	1 150	< 0,222	< LoB

12. PRECAUTIONS

- All reagents in the kit package are for *in vitro* diagnostic use only.
- 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 should 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.
- 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

1. Levay, P. F. & Viljoen, M. Lactoferrin: a general review. Haematologica 80, 252–67 (1995).
2. Gisbert, J. P., McNicholl, A. G. & Gomollon, F. Questions and answers on the role of fecal lactoferrin as a biological marker in inflammatory bowel disease. Inflammatory bowel diseases 15, 1746–54 (2009).
3. Uchida et al. Immunochemical detection of human lactoferrin in feces as a new marker for inflammatory gastrointestinal disorders and colon cancer. Clin. Biochem. 27, 259-64 (1994)

Used symbols:

 Temperature limitation

REF Catalogue Number

IVD In Vitro Diagnostic Medical Device

→REF To be used with

 Manufacturer

 Contains sufficient for <n> tests

LOT Lot number

 Use by

 Attention

 Consult instructions for use

 Consult specification data sheet