Distribuito in ITALIA da
Li StarFish S.r.l.
Via Cavour, 35
20063 Cernusco S/N (MI)
telefono 02-92150794
info@listarfish.it
www.listarfish.it

Manual

IDK® EDN ELISA

For the in vitro determination of EDN (eosinophil-derived neurotoxin) in stool, urine, serum and plasma

Valid from 2020-01-29

REF **K 6811**

Σ 96

+8°C +2°C

IVD

CE

Immundiagnostik AG, Stubenwald-Allee 8a, 64625 Bensheim, Germany
Tel.: +49 6251 70190-0 Fax: + 49 6251 70190-363
e.mail: info@immundiagnostik.com www.immundiagnostik.com

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1. INTENDED USE

This Immundiagnostik AG assay is an enzyme immunoassay intended for the quantitative determination of EDN (eosinophil-derived neurotoxin, also known as RNASE2 or eosinophil protein x [EPX]) in serum, plasma, urine and stool.

For *in vitro* diagnostic use only.

2. INTRODUCTION

EDN (eosinophil-derived neurotoxin, eosinophil protein x, EPX), a cationic glycoprotein, which is released by activated eosinophiles, has strong cytotoxic characteristics and plays a significant role in the prevention of virus infections. It is released by the eosinophile granules in places where eosinophiles are mainly found: in the skin, lungs, urogenital and gastrointestinal tract, that is, in the organs acting as an entry point for pathogens. The accumulation of EDN in the intestine is associated with inflammation and tissue damage.

Measuring of EDN in stool can serve as an objective parameter for a current clinical or sub-clinical chronic inflammation located in the gastrointestinal area. In the case of Colitis ulcerosa and Crohn's disease, the EDN measurement enables the evaluation of a disease's activity and the prediction of a relapse.

Indications

- Morbus Crohn
- Proof of a food allergy and incompatibility
- Assessment of an elimination diet
- Proof of damaged integrity of the intestinal mucous membrane (e. g. chronic inflammatory bowel disease, colon cancer)
- Proof of intestinal parasites / parasitoses

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity
K 6811	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	2 x 100 ml
K 6811	ASYBUF	Assay buffer, ready-to-use	1 x 50 ml
K 6811	STD	Standard, lyophilised (0; 0,25; 1; 4; 16 ng/ml)	2 x 5 vials

Cat. No.	Label	Kit components	Quantity
K 6811	CTRL 1	Control, lyophilised (see specification for range)	2 x 1 vial
K 6811	CTRL 2	Control, lyophilised (see specification for range)	2 x 1 vial
K 6811	CONJ	Conjugate concentrate, polyclonal peroxidase-labelled antibody	1 x 200 µl
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 purchase order 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
- Centrifuge, 3000 *g*
- 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 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. Before use, the STD and CTRL have to be reconstituted with **500 µl of ultrapure water** and mixed by gentle inversion to ensure complete reconstitution. Allow the vial content to dissolve for 10 minutes and then mix thoroughly. **Standards and controls** (reconstituted STD and CTRL) **can be stored at 2–8 °C for 4 weeks.**
- **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 storage

Raw stool can be stored for 72 hours at room temperature (15-30 °C) and 4 °C or for 8 weeks at -20 °C.

Stool extracts (1:100) can be stored for 1 day at room temperature (15–30 °C), for 5 days at 2–8 °C or for seven days at -20 °C. Avoid more than two 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 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 sample tube** with **1.5 ml extraction buffer** (1:2.5 diluted *IDK Extract®)* before using it with the sample. Important: Allow the 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 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

Stool samples

The supernatant of the extraction (dilution I) is diluted **1:4** with **wash buffer**. For example:

100 µl dilution I + **300 µl** wash buffer = **dilution II** (1:4)

This results in a **final dilution of 1:400***

* A dilution of 1:1000 is recommended for sample collectives with expected elevated values.

For analysis, pipet **100 µl of dilution II** per well.

Urine samples

We recommend to analyse urine collected within 24 hours, whereby the EDN concentration is expressed as mg/day. If a 24 h urine sample is not available, urine from a single time point can be analysed. In this case, the urinary creatinine should also be quantified, and the EDN results are presented as µg/mmol creatinine.

Within 30 min of urine collection, the urine is separated by centrifugation, twice for 10 min at 1350 *g* and 4 °C. The supernatant is then transferred to a new plastic tube.

Prior to analysis, the urine samples should be diluted **1:400** with assay buffer (ASYBUF).

For example:

10 µl sample + 1**90 µl** ASYBUF = **dilution I** (1:20)

15 µl dilution I + **285 µl** ASYBUF = **dilution II** (1:20)

This results in a **final dilution of 1:400**

For analysis, pipet **100 µl of dilution II** per well.

Serum/plasma samples

Fresh collected serum/plasma should be centrifuged within one hour. Store samples at -20 °C if not assayed on the same day. Lipemic or hemolytic samples may give erroneous results. Samples should be mixed well before assaying. We recommend duplicate analyses for each sample.

The serum/plasma samples should be diluted 1:40 with assay buffer (ASYBUF), prior to analysis.

10 µl sample + **390 µl** ASYBUF

This results in a **final dilution of 1:40**

For analysis, pipet **100 µl of the dilution** per well.

7. ASSAY PROCEDURE

Principle of the test

The assay utilises the two-site sandwich ELISA technique with two selected antibodies (monoclonal and polyclonal) that bind to human EDN.

Assay standards, controls and prediluted patient samples containing human EDN are added to wells of microplate that was coated with a high affine monoclonal anti-human EDN antibody. After the first incubation period, antibody immobilised on the wall of microtiter wells captures human EDN in the sample. Then a peroxidase-conjugated rabbit polyclonal anti-human EDN antibody is added to each microtiter well and a sandwich of capture antibody – human EDN – Peroxidase-conjugate is formed. Tetramethylbenzidine is used as a substrate for peroxidase. Finally, an acidic stop solution is added to terminate the reaction. The colour changes from blue to yellow. The intensity of the yellow colour is directly proportional to the concentration of EDN. A dose response curve of the absorbance unit (optical density, OD at 450 nm) vs. concentration is generated, using the values obtained from the standard. EDN present in the patient samples, is determined directly from this curve.

Test procedure

Prior to use in the assay, allow all reagents and samples to come to room temperature (15–30 °C) and mix well.

Mark the positions of standards/samples/controls on a protocol sheet.

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.

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 each **100 µl standards/samples/controls standards/controls/diluted 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 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.
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 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.
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, mix thoroughly.
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 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 programme used, the paired values should be evaluated manually.

Stool and urine samples

The obtained results have to be multiplied by the **dilution factor 400** or by **1 000** when a dilution of 1:1 000 has been used to get the actual concentrations.

Serum/plasma samples

The obtained results have to be multiplied by the **dilution factor 40** to get the actual concentrations.

In case **another dilution factor** has been used, multiply the obtained result with the dilution factor used to get the real concentration.

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 ranges

1 g stool is equivalent to 1 ml.

Stool (n = 53): 357.6 ng/ml (mean value)

Based on Immundiagnostik AG studies of evidently healthy persons (n = 53), a mean value of 357,64 ng/ml stool (standard deviation: 500,1 ng/ml) was estimated. The mean value + 2 SD (= 1357,8 ng/ml) should be considered as the preliminary upper limit of the test.

Urine (n = 50): 81.8 (26.7–164.2) µg/mmol Creatinine

Serum (n = 52): 26.4 (8.3–66.4) ng/ml

Plasma (n = 52): 18.1 (6.2–49.8) ng/ml

We recommend each laboratory to establish its own reference range.

11. PERFORMANCE CHARACTERISTICS

Accuracy – Precision

Repeatability (Intra-Assay); n = 28

The repeatability was assessed with 3 stool samples under **constant** parameters (same operator, instrument, day and kit lot).

Sample	Mean value [ng/ml]	CV [%]
1	1.53	7.2
2	3.73	8.4
3	0.50	8.4

Reproducibility (Inter-Assay); n = 20

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

Sample	Mean value [ng/ml]	CV [%]
1	1.84	12.4
2	3.62	6.8

Analytical sensitivity

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

Limit of blank, LoB 0.185 ng/ml

Accuracy – Trueness

The trueness states the closeness of the agreement between the result of a measurement and the true value of the measurand. Therefore, EDN spikes with known concentrations were added to 3 different stool samples.

Sample [ng/ml]	Spike [ng/ml]	Expected [ng/ml]	Obtained [ng/ml]	Recovery [%]
0.28	3.0	3.28	3.79	115.43
	4.0	4.28	4.61	107.64
	5.0	5.28	4.42	83.78
	8.0	8.28	7.39	89.24
0.67	1.5	2.17	2.18	100.41
	2.0	2.67	2.47	92.44
	2.5	3.17	2.96	93.38
	4.0	4.67	4.55	97.41
1.29	0.5	1.79	2.03	112.93
	1.5	2.79	3.22	115.32
	2.0	3.29	3.71	112.72
	3.5	4.79	5.52	115.04

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 EDN in serum plasma, stool and urine, the method has been demonstrated to be linear from 0.42 to 3.99 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	1:200	3.359	3.359	100.00
	1:400	1.679	1.736	103.36
	1:800	0.840	0.993	118.26
	1:1 600	0.420	0.558	132.99
B	1:200	3.990	3.990	100.00
	1:400	1.995	2.257	113.10
	1:800	0.998	1.156	115.84
	1:1 600	0.499	0.547	109.70

Analytical specificity

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

Substance tested	Concentration added [ng/ml]	Obtained [OD]	Fazit
Lactoferrin	240	0.009	< LoB
Secretory IgA	600	0.008	< LoB
Human albumin	800	0.007	< LoB
PMN elastase	10	0.008	< LoB

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 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*® and *IDK Extract*® are trademarks 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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Used symbols: