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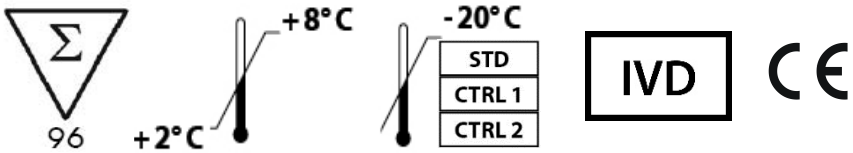
Manual

IDK® Tryptophan ELISA

For the in vitro determination of L-tryptophan in stool

Valid from 2019-07-17

REF **K 7729**



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1. INTENDED USE

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

2. INTRODUCTION

Tryptophan is an essential precursor of important substances regulating pain sensation and recovery:

- Serotonin and kynurenic acid: If their concentration in the mucosa is low, pain sensation in patients with colitis is more intense[1].
- Indolealdehyde, produced by lactobacilli in the gut, contributes to mucosal protection from inflammation and to colonization resistance to *candida albicans*[2].

Gupta *et al.* have shown that suppressed serum tryptophan levels correlate with high disease activity of IBD (Inflammatory Bowel Disease), and that tryptophan levels elevated as disease activity lessened[3]. A study with more than 500 patients with IBD confirmed the negative correlation between serum level of tryptophan and disease activity[4].

Lamas *et al.* have observed reduced tryptophan concentrations also in the stool of patients with IBD[5].

The IDK® tryptophan stool ELISA was developed to measure tryptophan levels in stool to assess the need for tryptophan supplementation.

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[1] Keszthelyi D et al. (2013). Decreased levels of kynurenic acid in the intestinal mucosa of IBS patients: Relation to serotonin and psychological state. *Journal of Psychosomatic Research*, 74(6), 501–504.

[2] Zelante T et al. (2013). Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. *Immunity,* 39(2), 372–385.

[3] Gupta NK et al. (2012). Serum analysis of tryptophan catabolism pathway: correlation with Crohn's disease activity. *Inflammatory Bowel Diseases*. 18(7):1214-20.

[4] Nikolaus S. et al. (2017). Increased Tryptophan Metabolism is Associated With Activity of Inflammatory Bowel Diseases. *Gastroenterology*. http://doi.org/10.1053/j.gastro.2017.08.028.

[5] Lamas B et al. (2016). CARD9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. *Nature Medicine*, 22(6), 598–605.*Inflammatory Bowel Diseases*. 18(7):1214-20.

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit Components	Quantity
K 7729	PLATE	Microtiter plate, pre-coated	12 x 8 wells
K 0006.C.100	WASHBUF A	Wash buffer concentrate, 10x	2 x 100 ml
K 7729	STD	Standards, ready-to-use ((0, 0.6, 2, 6, 20, 60 µM)	6 x 200 µl
K 7729	CTRL 1	Control, ready-to-use (see specification for range)	1 x 200 µl
K 7729	CTRL 2	Control, ready-to-use (see specification for range)	1 x 200 µl
K 7999.100	IDK® Amino Extract	Extraction buffer, ready-to-use	1 x 100 ml
K 7729	AB	L-tryptophan antibody, lyophilised	2 x 1 vial
K 7729	CONJ	Conjugate, ready-to-use	1 x 12 ml
K 7729	DER	Derivatisation reagent	100 mg
K 0008.07	DMSO	Dimethylsulfoxide (DMSO)	1 x 7 ml
K0011.28	ASYBUF	Assay buffer, ready-to-use	1 x 28 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 pipets 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.
- **Preparation of the wash buffer**: The **wash buffer concentrate (WASHBUF A)** has to be diluted with ultrapure water **1:10** before use (100 ml WASHBUF A + 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 A** is stable at **2-8 °C** until the expiry date stated on the label. **Wash buffer** (1:10 diluted WASHBUF A) can be stored in a closed flask at **2-8 °C for 1 month**.
- Store **standards and controls (STD/CTRL)** frozen at **-20 °C**. They are stable at -20 °until the expiry date stated on the label. Thaw before use in the test and mix well. Re-freeze standards and controls immediately after use.
- **DMSO** crystallizes at 4 °C. Before use, dissolve the crystals at room temperature or in a water bath.
- The **lyophilised derivatization reagent (DER)** is stable at **2-8 °C** until the expiry date stated on the label. Bring to room temperature before opening. Reconstitute the content of the vial (100 mg) with **6 ml DMSO**. Allow to dissolve for 10 minutes and mix thoroughly with a vortex-mixer. **The derivatization reagent** (dissolved DER) **can be stored at 2-8 °C for 2 months**. Bring to room temperature before reuse. Please note: DMSO attacks all plastics but not polypropylene products and laboratory glass.
- The **lyophilised L-tryptophan antibody (AB)** is stable at **2-8 °C** until the expiry date stated on the label. Dissolve the content of one vial of AB in **3 ml of wash buffer**. When more than one vial is to be used, combine the contents and mix prior to use. **L-tryptophan antibody** (dissolved AB) **can be stored at 2-8 °C for 2 months**.
- 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

Storage of samples

Stool extract is stable for 6 days at room temperature or 2-8 °C. For longer storage keep frozen at -20 °C. Avoid more than two freeze-thaw cycles.

Extraction of the stool sample

The extraction buffer (IDK® Amino Extract) is ready-to-use. We recommend the following sample preparation:

Stool Sample Application System (SAS) (Cat. No. K 6998SAS)

Stool sample tube – Instructios 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 750 µl buffer:

Applied amount of stool: 15 mg

Buffer volume (IDK® Amino Extract): 750 µl

Dilution factor: 1:50

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 homogenization using an applicator, inoculation loop or similar device.

b) Fill the **empty sample tube** with **750 µl of extraction buffer (IDK® Amino 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 the dipstick back into the tube. When putting the stick back into the tube, excess material will be stripped of, leaving 15 mg of sample to be diluted. Screw tightly to close the tube.

d) Shake 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.

To **25 µl of the stool extract** a derivatization reagent (DER) is added for derivatization of L-tryptophan (details are given in the sample preparation procedure).

7. ASSAY PROCEDURE

Principle of the test

This ELISA is designed for the quantitative determination of L-tryptophan. The assay is based on the method of competitive enzyme linked immunoassays.

The sample preparation includes the addition of a derivatisation reagent for L-tryptophan derivatisation. Afterwards, the treated samples and a polyclonal L-tryptophan antiserum are incubated in the wells of a microtiter plate coated with L-tryptophan derivative (tracer). During the incubation period, the target L-tryptophan in the sample competes with the tracer immobilised on the wall of the microtiter wells for the binding of the polyclonal antibodies.

During the second incubation step a peroxidase-conjugated antibody is added to each microtiter well to detect the L-tryptophan antibodies. After washing away the unbound components tetramethylbenzidine (TMB) is added as a peroxidase substrate. Finally, the enzymatic reaction is terminated by an acidic stop solution. The color changes from blue to yellow and the absorbance is measured in a photometer at 450 nm. The intensity of the yellow color is inverse proportional to the tryptophan concentration in the sample; this means, high L-tryptophan concentration in the sample reduces the concentration of tracer-bound antibodies and lowers the photometric signal. 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. L-Tryptophan, present in the patient samples, is determined directly from this curve.

Sample preparation procedure

Bring **all reagents and samples to room temperature** (15-30 °C) and mix well.

Derivatisation of standards, controls and extracted stool samples is carried out in single analysis in vials (e.g. 1.5 ml polypropylene vials).

We recommend preparing one derivatisation per standard, control and sample and transferring it in duplicate determinations into the wells of the microtiter plate.

1.	Add **25 µl standard** (STD)/ **control** (CTRL)/ **extracted stool sample** into the corresponding vials.
2.	Add **250 µl assay buffer** (ASYBUF) into each vial (STD, CTRL, sample).
3.	Add **50 µl derivatization reagent** into each vial (STD, CTRL, sample), **mix thoroughly** by repeated inversion or several seconds on a vortex mixer. Incubate for **45 min** at room temperature (15-30 °C) on a **horizontal shaker**.

2 x 50 µl of the derivatised standards/controls/samples are used in the ELISA as duplicates.

Test procedure

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

Take as many microtiter strips (PLATE) as needed from the kit. Store unused strips covered with foil at 2-8 °C. Strips are stable until expiry date stated on the label. Please note: Do not wash the plate.

4.	For the analysis in duplicate take **2 x 50 µl** of the **derivatised standards/ controls/samples** out of the vials and add into the respective wells of the microtiter plate.
5.	Add **50 µl L-tryptophan antibody** into each well of the microtiter plate.
6.	Cover the strips tightly with foil and incubate **overnight** (15-20 hours) **at 2-8 °C**.
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 fimly tapping the plate on absorbent paper.

8.	Add **100 µl conjugate** (CONJ) into each well.
9.	Cover the strips and incubate for **1 hour** at room temperature (15-30 °C) on a **horizontal shaker**.
10.	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 fimly tapping the plate on absorbent paper.
11.	Add **100 µl substrate** (SUB) into each well.
12.	Incubate for **12-18 min*** at room temperature (15-30 °C) in the **dark**.
13.	Add **100 µl stop solution** (STOP) into each well and mix well.
14.	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 (690 nm) as a reference.

* The intensity of the color change is temperature sensitive. We recommend observing the color change and stopping the reaction upon good differentiation.

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.

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 optical density and a logarithmic abscissa for 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 optical density and a linear abscissa for concentration.

3. Spline algorithm

We recommend a linear ordinate for optical density and a linear abscissa for concentration.

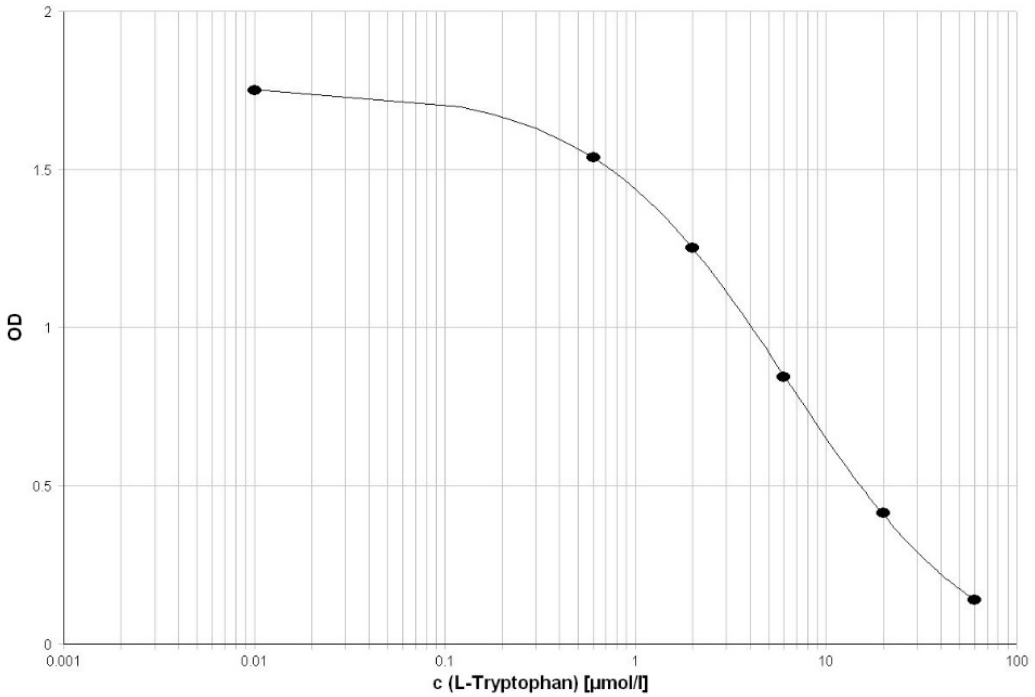
The plausibility of the duplicate values should be examined before the automatic evaluation of the results. If this option is not available with the program used, the duplicate values should be evaluated manually.

Stool samples

The obtained results have to be multiplied by the dilution **factor of 50** to get the actual concentrations (1 µmol/l = 1 nmol/g stool).

In case another dilution factor has been used, multiply the obtained result by the dilution factor used.

In the following, an example of a standard curve is given. Do not use it for the calculation of your results.



9. LIMITATIONS

Samples with concentrations above the measurement range can be further diluted with extraction buffer (IDK® Amino Extract) and re-assayed. Please consider this higher dilution when calculating the results.

Samples with concentrations lower than the measurement range 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".

Biotin interference

Samples containing a biotin concentration of < 12000 ng/ml show a change of the results of ≤ 25 %. Higher concentrations of biotin can lead to falsely low results. Patients taking > 5 mg biotin per day should wait at least 24 hours after taking biotin to have their samples collected. Results of patients taking biotin supplements or receiving a high-dose biotin therapy should generally be interpreted along with the total clinical picture.

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 samples are outside of the acceptable limits.

Reference Range

Based on internal studies with stool samples of apparently healthy persons (n = 62) a median of 128 nmol/g was estimated. For 3 % of this collective (5th percentile) an L-tryptophan concentration of less than 80 nmol/g was obtained.

This values correspond to the published results of Lamas *et al.* (2016, doi: 10.1038/nm.4102). In this study (n = 32) one out of 32 healthy persons had an L-tryptophan concentration less than 80 nmol/g (*i.e.* 3.1 %).

We recommend each laboratory to establish its own reference range.

11. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

Intra-assay (n = 12)

sample	L-tryptophan [nmol/g]	CV [%]
1	135.3	11.4
2	376.9	5.6

Inter-assay (n = 11)

sample	L-tryptophan [nmol/g]	CV [%]
1	141.8	11.5
2	343.0	6.5

Spiking recovery

Two samples were spiked with different L-tryptophan concentrations and measured in this assay. The mean recovery rate was 92.7 % (n = 2).

sample	spike [nmol/g]	expected [nmol/g]	measured [nmol/g]	recovery [%]
A			142.0	
	50	192.0	156.6	81.6
	150	292.0	276.1	94.6
	250	392.0	402.1	102.6
B			110.7	
	50	160.7	152.3	94.8
	150	260.7	207.1	79.4
	250	360.7	371.5	103.0

Dilution recovery

3 samples were diluted and measured in this assay. The mean recovery was 99.1 % (n = 2).

sample	dilution	expected [nmol/g]	measured [nmol/g]	recovery [%]
A	1:50		1.784	
	1:66.7	1.337	1.372	102.6
	1:100	0.892	0.871	97.6
B	1:50		2.655	
	1:66.7	1.990	1.820	91.5
	1:100	1.328	1.220	91.9
C	1:50		1.981	
	1:66.7	1.485	1.515	102.0
	1:100	0.991	1.080	109.0

Analytical sensitivity

The following values have been estimated based on the standard curve in consideration of the sample dilution factor of 50.

Limit of blank, LoB	30.2 nmol/g
Limit of detection, LoD	45.4 µmol/g
Limit of quantitation, LoQ	48.0 µmol/g

The evaluation was performed according to the CLSI guideline EP-17-A2. The specified accuracy goal for the LoQ was 20 % CV.

Specificity

The specificity of the antibody was tested by measuring the cross-reactivity against a range of compounds with structural similarity to tryptophan. The specificity is calculated in percent in relation to the tryptophan-binding activity:

5-HTP (5-hydroxytyptophan)	< 0.5 %
L-phenylalanine	< 0.1 %
L-tyrosine	< 0.1 %

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 color reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes

- The stop solution consists of diluted sulfuric 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 breathe 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*® 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 sent to Immundiagnostik AG along with a written complaint.

15. REFERENCES

1. Ciorba MA: Indoleamine 2,3 dioxygenase in intestinal disease. *Curr Opin Gastroenterol.* 2013 Mar;**29**(2):146-52. doi:10.1097/MOG.0b013e32835c9cb3.

2. Gupta NK, Thaker AI, Kanuri N, Riehl TE, Rowley CW, Stenson WF, Ciorba MA: Serum analysis of tryptophan catabolism pathway: correlation with Crohn's disease activity. *Inflamm Bowel Dis*. 2012 Jul;**18**(7):1214-20. doi:10.1002/ibd. 21849.

3. Keszthelyi D, Troost FJ, Jonkers DM, Kruimel JW, Leue C, Masclee AaM.: Decreased levels of kynurenic acid in the intestinal mucosa of IBS patients: Relation to serotonin and psychological state. *J Psychosom Res.* 2013;**74**(6):501-504. doi:10.1016/j.jpsychores.2013.01.008.

4. Lamas B, Richard ML, Leducq V, et al.: CARD9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. *Nat Med.* 2016;**22**(6):598-605. doi:10.1038/nm.4102.

5. Nikolaus S, Schulte B, Al-Massad N, Thieme F, Schulte DM, Bethge J, et al.: Increased Tryptophan Metabolism is Associated With Activity of Inflammatory Bowel Diseases. *Gastroenterology*. **2017**. doi:10.1053/j.gastro.2017.08.028

6. Thorburn AN, Macia L, Mackay CR. Diet, metabolites, and "western-lifestyle" inflammatory diseases. *Immunity*. 2014;**40**(6):833-842. doi:10.1016/j.immuni. 2014.05.014.

7. Zelante T, Iannitti RG, Cunha C, et al.: Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. *Immunity*. 2013;**39**(2):372-385. doi:10.1016/j.immuni.2013.08. 003.

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