Manual

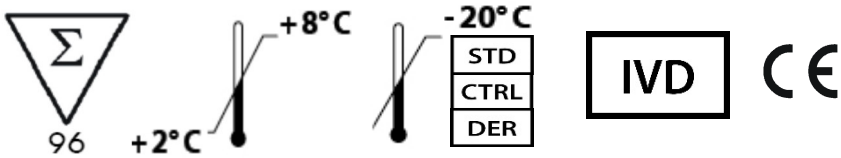
Distribuito in ITALIA da
Li StarFish S.r.l.
Via Cavour, 35
20063 Cernusco S/N (MI)
telefono 02-92150794
info@listarfish.it
www.listarfish.it

GABA ELISA

For the in vitro determination of GABA in stool

Valid from 2020-06-17

REF **K 7009**



Immundiagnostik AG, Stubenwald-Allee 8a, 64625 Bensheim, Germany
Tel.: + 49 6251 70190-0 Fax: + 49 6251 70190-363

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1. INTENDED USE

This Immundiagnostik AG assay is intended for the quantitative determination of γ-aminobutyric acid (GABA) in stool. For *in vitro* diagnostic use only.

2. INTRODUCTION

Gamma-aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the central and also peripheral nervous system in humans.

Recent publications indicate an important role for GABA in irritable bowel syndrome (IBS). Here, GABA develops its inhibitory effect and alleviates visceral pain (Loeza-Alcocer et al. 2019; Icenhour et al. 2019). Accordingly, a low GABA level indicates high pain sensitivity in IBS (Aggarwal et al. 2018).

GABA formed by certain lactobacilli and bifidobacteria – such as L. plantarum, L. rhamnosus, L. acidophilus, and B. adolescentis, B. longum, B. bifidum, B. breve – can act on the GABA receptors in the intestine and can have beneficial effects on IBS symptoms via the brain-gut axis (Mezzasalma et al. 2016).

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit Components	Quantity
K 7009	PLATE	Microtiter plate, pre-coated	12 x 8 wells
K 7009	STD	Standards ready-to-use, (0, 0.1, 0.3, 1, 3, 10 µg/ml)	6 x 250 µl
K 7009	CTRL 1	Control, ready-to-use (see specification for range)	1 x 250 µl
K 7009	CTRL 2	Control, ready-to-use (see specification for range)	1 x 250 µl
K 0001.C.100	WASHBUF	Wash buffer concentrate, 10x	2 x 100 ml
K 7999.100	IDK® Amino Extract	Extraction buffer, ready-to-use	1 x 100 ml
K 7009	ASYREAG	Assay reagent, lyophilised	1 vial
K 7009	CONJ	Conjugate, peroxidase-labelled, ready-to-use	1 x 12 ml
K 0012.50	DERBUF	Reaction buffer, ready-to-use	1 x 50 ml
K 7009	DER	Derivatisation reagent, lyophilised	3 x 1 vial

K 0008.04	DMSO	Dimethylsulfoxide (DMSO)	1 x 4 ml
K 0013.50	CODIL	Dilution buffer after derivatisation, ready-to-use	1 x 50 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. **Prepare only the appropriate amount necessary for each run**. The kit can be used up to 3 times within the expiry date stated on the label.
- **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**.

- Store **standards and controls (STD/CTRL)** frozen at **-20 °C**. They are stable at -20 °C until the expiry date stated on the label. Thaw before use in the test and mix well. Re-freeze standards and controls after use.
- **DMSO** crystallises at 2-8 °C. Before use, bring to room temperature to dissolve the crystals.
- Store the **lyophilised derivatisation reagent (DER)** frozen at **-20 °C**. Bring to room temperature before opening. Reconstitute one vial of DER with **1 ml DMSO**. Allow to dissolve for 10 min and mix thoroughly with a vortex-mixer. The derivatisation reagent must be **prepared immediately before use**. When more than one vial is to be used, combine the contents and mix prior to use. Discard any rest of the reagent after use. Please note: DMSO attacks all plastics but not polypropylene products and laboratory glass.
- The **lyophilised assay reagent (ASYREAG)** is stable at **2-8 °C** until the expiry date stated on the label. Reconstitute the ASYREAG with **6 ml of wash buffer**. **Assay reagent** (reconstituted ASYREAG) **can be stored at 2-8 °C for 1 month.**
- 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

Extraction of the stool samples

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 0.75 ml buffer:

Applied amount of stool:	15 mg
Buffer volume (IDK® Amino Extract):	0.75 ml
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 stool sample tube** with **0.75 ml extraction buffer (IDK® Amino Extract)** before using it with the sample.

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.

Dilution I: 1:50

Dilution of samples

Dilute the supernatant of the sample extraction (dilution I) **1:2 with extraction buffer (IDK® Amino Extract).** For example:

100 µl supernatant (dilution I) + **100 µl IDK® Amino Extract**, mix well
= **1:2** (**dilution II**).
This results in a final dilution of **1:100**.

To **50 µl of dilution II** a derivatisation reagent is added for derivatisation of GABA (see derivatisation procedure).

Sample storage

Raw stool is stable for up to 3 days at room temperature. For longer storage keep frozen at -20 °C.

Stool extract is stable for one week at room temperature. For longer storage keep frozen at -20 °C.

7. ASSAY PROCEDURE

Principle of the test

This ELISA is desiged for the quantitative determination of GABA in stool. The assay is based on the method of competitive enzyme linked immunoassays.

The sample preparation includes the addition of a derivatisation reagent for GABA derivatisation. Afterwards, the treated samples are incubated in wells of a microtiter plate coated with a polyclonal antibody against GABA-derivative, together with an assay reagent containing GABA-derivative (tracer).

During the incubation period, the target GABA in the sample competes with the tracer for the binding of the polyclonal antibodies on the wall of the microtiter wells.

During the second incubation step, a peroxidase conjugate is added to each microtiter well to detect the tracer. 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 colour 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 GABA concentration in the sample; this means, high GABA concentration in the sample reduces the concentration of antibody-bound tracer 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. GABA present in the patient samples is determined directly from this curve.

Derivatisation procedure

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

Derivatisation of standards, controls and diluted samples is carried out 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 **50 µl standard** (STD)/ **control** (CTRL)/ **sample from dilution II** in the corresponding vials.
2.	Add **500 µl reaction buffer** (DERBUF) into each vial (STD, CTRL, sample), mix well.

3.	Add **25 µl** of freshly prepared **derivatisation reagent** into each vial and **mix thoroughly** by repeated inversion or several seconds on a vortex mixer. Incubate for **1 hour** at room temperature (15-30 °C) on a **horizontal shaker**.
4.	Add **500 µl dilution buffer** (CODIL) into each vial, mix well and incubate for **30 min** at room temperature (15-30 °C) on a horizontal **shaker**.

2 x 50 µl of the derivatised standards, controls and 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 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.

5.	**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.
6.	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.
7.	Add **50 µl assay reagent** into each well.
8.	Cover the strips tightly with foil and incubate **overnight at 2-8°C**.
9.	Discard the content of each well and wash **5 times** with **250 µl of wash buffer**. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.
10.	Add **100 µl conjugate** (CONJ) into each well.
11.	Cover the strips and incubate for **1 hour** at room temperature (15-30 °C) on a **horizontal shaker**.
12.	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.

13.	Add **100 µl substrate** (SUB) into each well.
14.	Incubate for **10-20 min*** at room temperature (15-30 °C) in the **dark**.
15.	Add **100 µl stop solution** (STOP) into each well and mix well.
16.	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 to observe the color change and to stop 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.

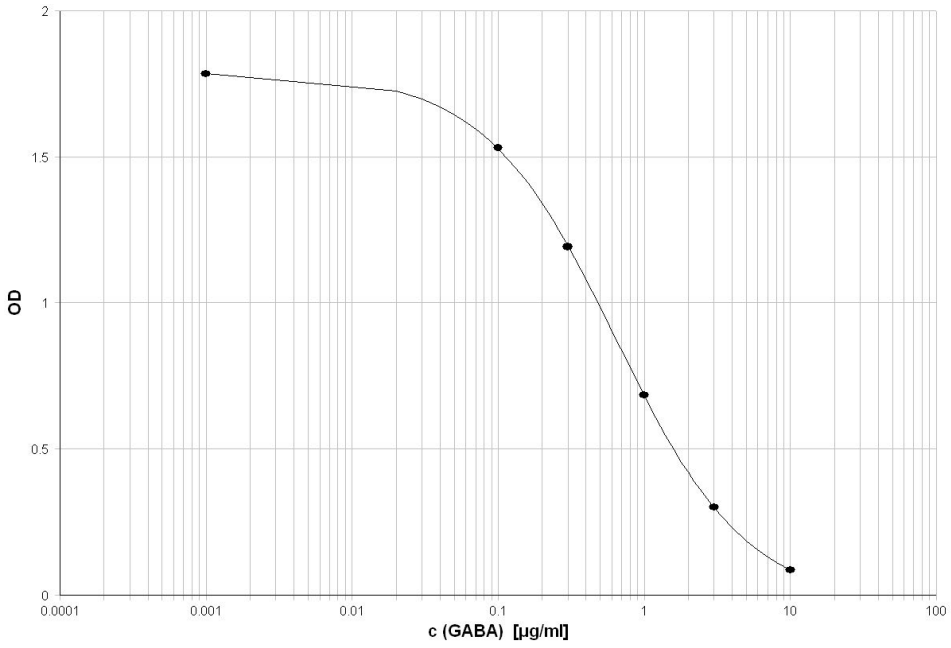
The plausibility of the duplicate values should be examined before the automatic evaluation of the results. If this option is not available with the program used, the duplicate values should be evaluated manually.

Stool samples

The obtained results have to be multiplied by the **dilution factor of 100** (1 µg/ml = 1 µg/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 dilution factor 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:

Analytical sensitivity × *sample dilution factor to be used*

Analytical sensitivity see chapter “Performance Characteristics”.

Biotin interference

Samples containing a biotin concentration of ≤ 22.3 µg/g stool show a change of the results of < 25 %. Higher concentrations of biotin can lead to false 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 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 samples of apparently healthy persons, a median of 27.4 µg/ml was estimated (n = 40). For 10 % of this collective a GABA concentration of less than 13.6 µg/ml was obtained (1 µg/ml =1 µg/g Stool).

We recommend each laboratory to establish its own reference range.

11. PERFORMANCE CHARACTERISTICS

Accuracy – Precision

Repeatability (Intra-Assay); n = 16

The repeatability was assessed with 4 samples under constant parameters (same operator, measurement system, day and kit lot).

sample	mean value [µg/ml]	CV [%]
1	9.7	11.7
2	23.4	7.8
3	61.3	6.4
4	113.5	3.0

Reproducibility (Inter-Assay); n = 11

The reproducibility was assessed with 4 samples under varying parameters (different operators, measurement systems, days and kit lots).

sample	mean value [µg/ml]	CV [%]
1	13.7	9.6
2	41.7	7.3
3	113.5	5.3
4	334.3	6.2

Accuracy – Trueness

The trueness states the closeness of the agreement between the result of a measurement and the true value of the measurand. Therefore, 3 high level samples (spikes) were added to 3 low level samples. The expected values result from the mean of sample and spike.

sample [µg/ml]	spike [µg/ml]	expected [µg/ml]	obtained [µg/ml]	recovery [%]
8.9	94.4	51.7	55.5	**107.5**
	334.4	171.7	166.4	**96.9**
	293.1	151.0	132.3	**87.6**
20.1	94.4	57.3	63.6	**111.1**
	334.4	177.3	165.2	**93.2**
	293.1	156.6	134.4	**85.8**
34.5	94.4	64.5	71.6	**111.1**
	334.4	184.5	190.0	**103.0**
	293.1	163.8	139.7	**85.3**

Analytical sensitivity

The zero-standard was measured 80 times. The detection limit was set as B_0 - 2 SD and estimated to be 0.046 µg/ml. The value has been estimated based on the concentrations of the standard curve without considering any sample dilution factor.

Specificity

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

β-alanine	≤ 0.02 %
β -aminobutyric acid	≤ 0.02 %
α-aminobutyric acid	≤ 0.005 %
glycine	≤ 0.006 %
glutamine	0.000 %

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 with a serial dilution of 4 extracted stool samples.

For GABA in stool, the method has been demonstrated to be linear from 0.078 to 2.427 µg/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 [µg/ml]	dilution	expected [µg/ml]	obtained [µg/ml]	recovery [%]
1	1:100		2.427	
	1:150	1.618	1.444	89.2
	1:200	1.214	1.152	94.9
	1:250	0.971	0.825	85.0
	1:300	0.809	0.731	90.4
	1:400	0.607	0.649	107.0
2	1:100		0.651	
	1:150	0.434	0.407	93.8
	1:200	0.326	0.299	91.9
	1:250	0.260	0.235	90.2
	1:300	0.217	0.206	94.9
	1:400	0.163	0.190	116.7

3	1:100		0.310	
	1:150	0.207	0.213	103.1
	1:200	0.155	0.171	110.3
	1:250	0.124	0.106	85.5
	1:300	0.103	0.094	91.0
	1:400	0.078	0.090	116.1
4	1:100		0.240	
	1:150	0.160	0.154	96.3
	1:200	0.120	0.097	80.8
	1:250	0.096	0.079	82.3
	1:300	0.080	0.064	80.0

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 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.
- 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. LITERATURE

1. Aggarwal S, Ahuja V, Paul J (2018) Dysregulation of GABAergic signalling contributes in the pathogenesis of diarrhea predominant irritable bowel syndrome. *Journal of Neurogastroenterology and Motility* **24**(3):422-430. doi: 10.5056/jnm17100
2. Icenhour A, Tapper S, Bednarska O, Witt ST, Tisell A, Lundberg P, Elsenbruch S, Walter S. (2019) Elucidating the putative link between prefrontal neurotransmission, functional connectivity, and affective symptoms in Irritable Bowel Syndrome. *Scientific Reports* **9**(1):13590. doi:10.1038/s41598-019-50024-3.
3. Loeza-Alcocer E, McPherson TP, Gold MS (2019) Peripheral GABA receptors regulate colonic afferent excitability and visceral nociception. *Journal of Physiology* **597**(13):3425-3439. doi: 10.1113/JP278025
4. Mezzasalma V, Manfrini E, Ferri E, Sandionigi A, La Ferla B, Schiano I, et al. (2016) A Randomized, Double-Blind, Placebo-Controlled Trial: The Efficacy of Multispecies Probiotic Supplementation in Alleviating Symptoms of Irritable Bowel Syndrome Associated with Constipation. *BioMed Research International* 1-10. doi: 10.1155/2016/4740907

5. Patterson E, Ryan PM, Wiley N, Carafa I, Sherwin E, Moloney G, Franciosi E, et al. (2019) Gamma-aminobutyric acid-producing lactobacilli positively affect metabolism and depressive-like behaviour in a mouse model of metabolic syndrome. *Scientific Reports* **9**(1):16323. doi: 10.1038/s41598-019-51781-x

6. Pokusaeva K, Johnson C, Luk B, Uribe G, Fu Y, Oezguen N, Matsunami RK, et al. (2017) GABA-producing Bifidobacterium dentium modulates visceral sensitivity in the intestine. *Neurogastroenterology & Motility* **29**(1):e12904. doi: 10.1111/nmo.12904

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