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User's Manual

Gamma-aminobutyric acid (GABA) ELISA Kit

REF **DEIASL174**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

This GABA assay is intended for the quantitative determination of γ-aminobutyric acid (GABA) in stool. For research use only.

General Description

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).

Principles of Testing

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.

Reagents And Materials Provided

Label	Kit Components	Quantity
PLATE	Microtiter plate	12 x 8 wells
STD	Standards ready-to-use, (0, 0.1, 0.3, 1, 3, 10 µg/ml)	6 x 250 µl
CTRL 1	Control, lyophilised (see specification for range)	1 x 250 µl
CTRL 2	Control, lyophilised (see specification for range)	1 x 250 µl
WASHBUF	Wash buffer concentrate, 10x	2 x 100 ml
Amino Extract	Extraction buffer, ready-to-use	1 x 100 ml
ASYREAG	Assay reagent, lyophilised	1 vial
CONJ	Conjugate, peroxidase-labelled, ready-to-use	1 x 12 ml
DERBUF	Reaction buffer, ready-to-use	1 x 50 ml
DER	Derivatisation reagent, lyophilised	3 x 1 vial
DMSO	Dimethylsulfoxide (DMSO)	1 x 4 ml
CODIL	Dilution buffer after derivatisation, ready-to-use	1 x 50 ml
SUB	Substrate (tetramethylbenzidine), ready-to-use	1 x 15 ml
STOP	Stop solution, ready-to-use	1 x 15 ml

Materials Required But Not Supplied

1. Ultrapure water
2. Stool sample application system
3. Calibrated precision pipets and 10-1000 µl single-use tips
4. Foil to cover the microtiter plate
5. Horizontal microtiter plate shaker
6. Multi-channel pipets or repeater pipets
7. Vortex
8. Standard single-use laboratory glass or plastic vials, cups, etc.
9. Microtiter plate reader

Specimen Collection And Preparation

Extraction of the stool samples

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

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 (Amino Extract): 0.75 ml

Dilution factor: 1:50

Please follow the instructions for the preparation of stool samples using the Stool sample tube 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 (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.

Sample Dilution

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

100 µl supernatant (dilution I) + 100 µl 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.

Reagent Preparation

1. 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.
2. 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.
3. 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.
4. DMSO crystallises at 2-8 °C. Before use, bring to room temperature to dissolve the crystals.
5. 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.

6. 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.
7. All other test reagents are ready-to-use. Test reagents are stable until the expiry date (see label) when stored at 2-8 °C.

Assay Procedure

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.

Notes: 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.

Quality Control

Creative Diagnostics 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.

Calculation

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

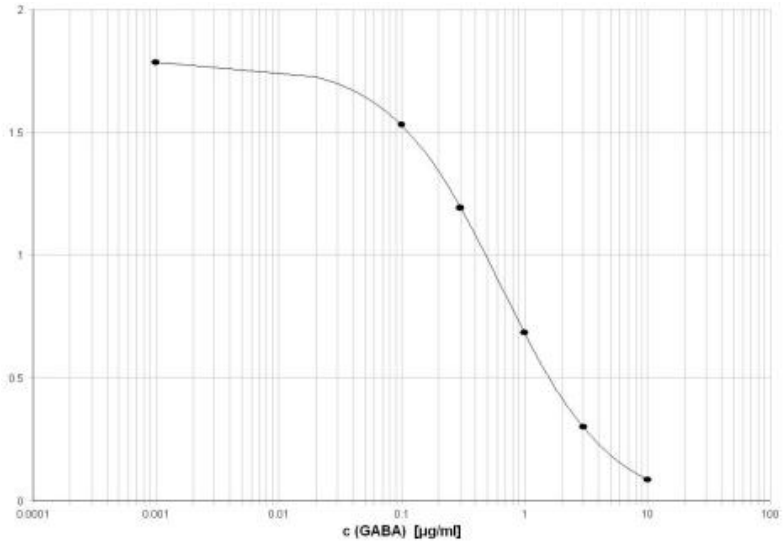
We recommend a linear ordinate for optical density and a linear abscissa for concentration.

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.

Typical Standard Curve

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.



Reference Values

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.

Precision

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

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

Sensitivity

The zero-standard was measured 80 times. The detection limit was set as B0 - 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.

Interferences

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.

Limitations

Samples with concentrations above the measurement range can be further diluted with extraction buffer (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".