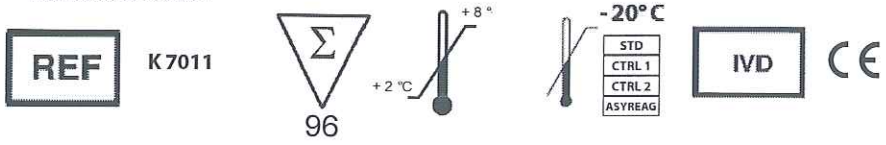
Manual

Gliadorphin (Gliadomorphin) ELISA Kit

For the determination of Gliadorphin in urine

Valid from 11.07.2013



1. Intended use

This ELISA Kit is intended for the determination of Gliadorphin in urine. It is for *in vitro* diagnostic use only.

2. Introduction

Gliadorphin is a 7 amino acids peptide which is formed during digestion of the gliadin component of the gluten protein. Gluten-derived peptides bind to opioid receptors in the brain and exhibit morphine-like effects, for example like heroin. These compounds have been shown to react with areas of the brain which are involved in speech and auditory integration.

Urine samples from people with autism, schizophrenia, and celiac disease contain high amounts of gliadorphin. It is suspected that this peptide may also be elevated in other disorders such as chronic fatigue, fibromyalgia, and depression. Symptom remission has been observed after exclusion of wheat and dairy products from the diet.

Indication

- Autism
- Schizophrenia
- Celiac disease

3. Principle of the test

The assay is based on the method of competitive enzyme linked immunoassays. Reaction buffer is used for sample preparation. Afterwards, the diluted samples and a Gliadorphin-derivative (tracer) are incubated in the microtiter plate wells coated with a Gliadorphin-antiserum. During the incubation, the target Gliadorphin in the sample competes with the tracer for the binding on the polyclonal antibodies immobilized on the wall of the microtiter wells. Gliadorphin in the sample displaces the tracer bound to the antibodies. Therefore, the concentration of the antibody-bound tracer is inverse proportional to the Gliadorphin concentration in the sample. During the second incubation step, a peroxidase-conjugated antibody, which binds to the tracer, is added to each microtiter well. After washing the unbound components, the peroxidase substrate tetramethylbenzidine (TMB) is added. Finally, the enzymatic reaction is terminated by an acidic stop solution. The color changes from blue to yellow and the absorbance is measured in the photometer at 450 nm. The intensity of the yellow color is inverse proportional to the Gliadorphin concentration in the sample; this means high Gliadorphin

concentration in the sample reduces the concentration of the antibody-bound tracer and lowers the photometric signal.

A dose response curve of absorbance unit (optical density, OD at 450 nm) vs. concentration is generated using the values obtained from the standard. Gliadorphin present in the patient samples is determined directly from this curve. The ELISA results are normalized to the creatinine concentration of the urine sample. For this reason, a parallel determination of the creatinine concentration is required.

4. Material supplied

Cat. No	Content	Kit Components	Quantity
K7011MTP	PLATE	One holder with precoated strips	12 x 8 wells
K7011ST	STD	Standards, lyophilized	6 x 1 vial
K7011KO1 K7011KO2	CTRL 1 CTRL 2	Controls, lyophilized	2 x 1 vial
K7011WP	WASHBUF	ELISA wash buffer concentrate 10x	2 x 100 ml
K7011CSP	2.ABDIL	Conjugate stabilizing buffer, ready to use	12 ml
K7011AR	ASYREAG	Assay reagent (gliadorphin derivative), lyophilized	2 x 1 vial
K7011K	2.AB	POD antibody (concentrate 200x)	60 µl
K7011RP	DERBUF	Reaction buffer, ready to use	50 ml
K7011TMB	SUB	TMB substrate (Tetramethylbenzidine), ready to use	15 ml
K7011AC	STOP	ELISA stop solution, ready to use	15 ml

5. Material Required but not supplied

- Ultra pure water*
- Precision pipettors and disposable tips to deliver 10-1000 µl
- Foil to cover the microtiter plate
- Horizontal microtiter plate shaker
- A multi-channel dispenser or repeating dispenser
- Centrifuge capable of 3000 x g

- Vortex-Mixer
- Standard laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader at 450 nm or 405nm

* Immundiagnostik AG recommends the use of Ultra Pure 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).

6. Preparation and storage of reagents

- To run assay more than once ensure that reagents are stored at conditions stated on the label. **Prepare only the appropriate amount necessary for each assay**. The kit can be used up to 2 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.
- Dilute the **wash buffer concentrate (WASHBUF)** with ultra pure water **1:10** before use (**100 ml WASHBUF + 900 ml ultra pure water**), mix well. Crystals may occur due to high salt concentration in the stock solution. The crystals must be redissolved at room temperature or at 37°C using a water bath before dilution. The WASHBUF is stable at 2-8°C until the expiry date stated on the label. Diluted buffer solution can be stored in a closed flask at **2-8°C for one month**.
- **Standards (STD)** and **controls (CTRL)** are lyophilized and must be reconstituted in **1 ml** of **reaction buffer (DERBUF)** per vial. Put the vials on a horizontal shaker for 5 min. Store the reconstituted STD and CTRL frozen at **-20°C**, re-freeze immediately after use. They can be re-frozen up to 2 times.
- The **assay reagent (ASYREAG)** (gliadorphin derivative) is lyophilized and has to be reconstituted in **2 ml** of **diluted wash buffer** per vial. Put the vial on a horizontal shaker for 5 min. The ELISA kit can be separated into two performances by providing two ASYREAG vials. When more than one vial is to be used, combine the reconstituted solutions in a separate vial and mix prior to use. Reconstituted assay reagent can be stored at **-20°C** for **one month.**
- Dilute the **POD antibody (2.AB) 1:200** with conjugate stabilizing buffer (2.ABDIL) (e.g. **55 µl 2.AB + 11 ml 2.ABDIL**, prepare only the required amount). Undiluted POD antibody (2.AB) is stable at 2-8°C until the expiry date stated on the label. Diluted POD antibody (2.AB) can be stored at **2-8°C** for **1 week**.

- All other test reagents can be stored at 2-8°C. Test reagents are stable until the expiry date (see label of test package).

7. PRECAUTIONS

- For *in vitro* diagnostic use only.
- Kit reagents contain sodium azide or thimerosal as bactericides. Sodium azide and thimerosal are toxic. Substrates for the enzymatic color reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
- Stop solution is composed of sulfuric acid, which is a strong acid. Even diluted, it still must be handled with care. It can cause acid burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spills should be wiped out immediately with copious quantities of water.
- Reagents should not be used beyond the expiration date shown on kit label.

8. SAMPLE AND TEST PREPARATION

- Urine is suited for this test system (recommendation: early morning urine).
- Samples should be sent cooled; but they are stable for 24 h at room temperature.
- Samples must be **diluted** at least **1:10** in reaction buffer (DERBUF) prior to analyses (see test procedure).
- Samples are stable for two days at 2-8°C. For longer storage samples should be frozen at -20°C.

9. ASSAY PROCEDURE

Procedural notes

- The assay should always be performed according to the enclosed manual.
- Do not interchange different lot numbers of any kit component within the same assay.
- Quality control guidelines should be observed.
- Incubation time, incubation temperature, and pipetting volumes of the different components are defined by the producer. Any variations of the test procedure that are not coordinated with the producer may influence the test

results. Immundiagnostik AG can therefore not be held reliable for any damage resulting from this.

- The ELISA results are normalized to the creatinine concentration of the urine sample. For this reason, a parallel determination of the creatinine concentration is required.

Test procedure

Dilute samples in **reaction buffer (DERBUF)** by factor **1:10**,
i.e. **50 µl** sample and **450 µl** DERBUF.

1.	Bring all reagents and samples to **room temperature** (15-30°C)
2.	Mark the positions of standards (STD)/ controls (CTRL)/ samples (SAMPLE) in duplicate on a protocol sheet.
3.	Take as many microtiter strips (PLATE) as needed from kit. Store unused strips covered at 2-8°C. Strips are stable until the expiry date stated on the label.
4.	Wash each well **5 times** with **250 µl** of diluted **wash buffer**. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.
5.	For the analysis in duplicate, pipette **2 x 50 µl of** reconstituted **standards (STD)/ controls (CTRL)/ diluted samples (SAMPLE)** into the respective wells of the microtiter plate (PLATE).
6.	Add **50 µl** reconstituted **assay reagent (ASYREAG)** (gliadorphin derivative) into each well, cover the plate tightly.
7.	Incubate overnight **(15-20 hours)** at **2-8°C.**
8.	Aspirate the contents of each well. Wash each well **5 times** with **250 µl** of diluted **wash buffer**. After the final washing step the inverted microtiter plate should be firmly tapped on absorbent paper.
9.	Add **100 µl** of diluted **POD antibody** (**2.AB**) into each well.
10.	Cover the plate and incubate for **1 hour** at **room temperature** (15-30°C) on a horizontal shaker (180-240 rpm)

11. Aspirate the contents of each well. Wash each well **5 times** with **250 µl** of diluted **wash buffer**. After the final washing step the inverted microtiter plate should be firmly tapped on absorbent paper.
12. Add **100 µl** of **TMB substrate (SUB)** into each well.
13. Incubate for **12-25 min** at **room temperature** (15-30°C) in the dark*.
14. Add **50 µl** of **stop solution (STOP)** into each well, mix thoroughly.
15. Determine **absorption** immediately with an ELISA reader at **450 nm**. If the highest extinction of the standards **(STD)** is above the range of the photometer, absorption must be measured immediately at **405 nm** and the obtained results used for evaluation. If possible, the extinctions from each measurement should be compared with extinctions obtained at a reference wavelength, e. g. 595 nm, 620 nm, 630 nm, 650 nm and 690 nm can be used.

* The intensity of the color change is temperature sensitive. We recommend to observe the color change and to stop the reaction upon good differentiation.

10. Evaluation of Results

If the test is performed in strict compliance with the manufacturer's instructions, i.e. with the exact volumes for standards, controls and samples/sample treatment, standards, controls and samples are equally diluted. Therefore, **no dilution factor is required for calculation of the 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 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 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.

Plausibility of the measured pairs of values should be examined before automatically evaluating the results. If this option is not available within the used program, the pairs of values should be controlled manually.

The ELISA results are normalized to the creatinine concentration of the urine sample.

$$\text{Concentration}_{\text{Sample}}\,[\text{ng/µmol Creatinine}] = \frac{\text{Gliadorphin Concentration}_{\text{Sample}}\,[\text{ng/ml}]}{\text{Creatinine Concentration}_{\text{Sample}}\,[\text{mmol/l}]}$$

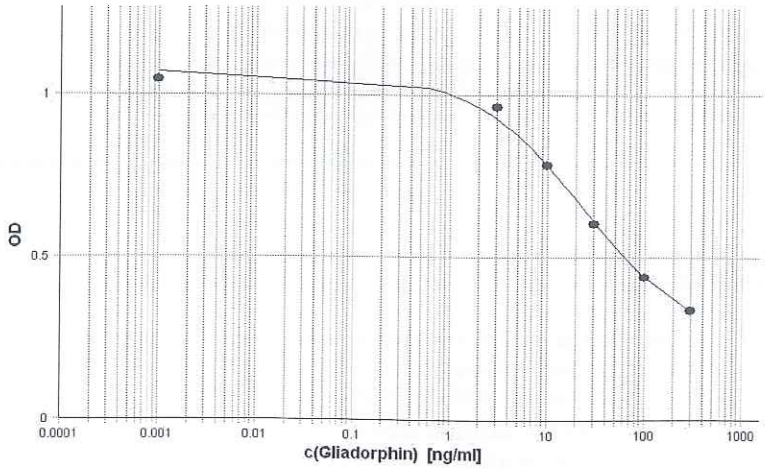
Controls

Control samples should be analyzed with each run. Results, generated from the analysis of the 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.

The concentration of controls and patient samples can be determined directly from calibration curve in ng/ml if a dilution of 1:10 has been used.

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

Example of a calibration curve



Expected values

Based on internal studies of evidently healthy persons (n=167) a normal range of

0 - 0.9 ng gliadorphin / µmol creatinine

was estimated.

We recommend each laboratory to develop its own normal range. The values mentioned above are indicative only and can deviate from other published data.

11. PERFORMANCE CHARACTERISTICS

Cross reactivity

Casomorphin: No cross reactivity was observed with casomorphin at concentration up to 1000 ng/ml in urine.

Gliadin: No cross reactivity was observed with gliadin at concentration up to 10 µg/ml in urine.

Precision and reproducibility

Intra-Assay (n=6)		
Sample	Gliadorphin [ng/ml]	Coefficient of variation (CV) [%]
1	6.45	12.8
2	22.6	7.8

Inter-Assay (n=4)		
Sample	Gliadorphin [ng/ml]	Coefficient of variation (CV) [%]
1	6.3	12.7
2	21.8	9.3

Sensitivity

The detection limit was set as B_0 - 1SD. The zero-standard was measured 12 times.

Sample	Gliaorphin mean value [OD]	Standard Deviation (SD)	Detection limit [ng/ml]
Zero standard	3	0.12	2

Linearity

The linearity of the ELISA was determined by the dilution of a urine sample spiked with 32 ng/ml Gliadorphin. The mean linearity was 132% (n=6).

Dilution	Measured [ng/ml]	Expected [ng/ml]	Recovery [%]
1:2	24	16	150
1:4	9,1	8	114

12. LIMITATIONS

Gliadorphin can only be determined in urine samples.

13. REFERENCES

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14. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and put on the market according to the IVD guidelines of 98/79/EC.
- All reagents in the test package are for *in vitro* diagnostic use only.
- Guidelines for medical laboratories should be observed.
- Incubation time, incubation temperature and pipetting volumes of the different components are defined by the producer. Any variations of the test procedure that are not coordinated with the producer may influence the results of the test. Immundiagnostik AG can, therefore, not be held reliable for any damage resulting from this.

Used symbols: