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GlutenTox® ELISA Competitive G12

Kit for the determination of gluten, suitable for hydrolyzed food samples

REF KIT3012

GlutenTox®•ELISA
Competitive G12

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1. Intended Use

GlutenTox ELISA Competitive is an immunosorbent assay for the determination of the immunotoxic fraction of gluten that is harmful to celiac patients. This test is suitable for quantifying gluten in hydrolyzed foods.

2. Introduction

Celiac disease is a disorder that damages the small intestine causing the atrophy of the intestinal villi, which interferes with the absorption of nutrients such as proteins, lipids, carbohydrates, mineral salts and vitamins. This disease is caused by an inappropriate response of the immune system to gluten (a mix of proteins found in cereals) from wheat, barley, rye and, to a lesser extent, from oat [ref. 1 and 2], leading to diarrhea, vitamin and mineral deficiencies, anemia and thin bones (osteoporosis). Celiac disease affects people of all ages.

Currently, the only treatment for celiac disease sufferers is a strict, lifelong gluten-free diet that presents great difficulties because gluten, in addition to being present in many foods, may also be found in food additives and preservatives.

According to the Codex Alimentarius Commission and the EC Regulation 41/2009 on the composition and labeling of foodstuffs suitable for people intolerant to gluten, food can be considered “gluten-free” if its gluten content does not exceed 20 parts per million (ppm*).

* Milligrams of gluten per kilo of food.

3. Test basis

GlutenTox ELISA Competitive G12 is a quantitative enzyme-linked immunosorbent assay (ELISA) designed for the determination of the immunotoxic fraction of gluten, including hydrolyzed gluten, in food samples.

In all methods used for gluten analysis in a given sample, the gluten first has to be extracted from the sample’s matrix. Extraction is one of the most critical points of the testing process. The extraction solution provided in this kit, Universal Gluten Extraction Solution (UGES), is suited for all types of food thanks to the combination of denaturing agents, reducing agents and solubilizers.

After the extraction, the food extract is incubated with the G12 anti-gliadin antibody that specifically recognizes the most toxic or immunogenic fraction of gluten [ref. 3]. After the washing steps, the addition of a conjugated to HRP (horseradish peroxidase) will allow to measure the signal in the presence of a substrate. The competitive ELISA is an indirect method. The lower the concentration of gluten present in the sample, the more intense the signal will be.

The Competitive ELISA method can be used to analyze both large and small antigens. However, in contrast to other types of ELISA, it is an ideal method when the antigen is too small to be detected by two antibodies simultaneously; this is often the case of hydrolyzed peptides. Moreover, this technique is commonly used for the analysis of substances at very low concentrations. This, together with the high specificity and sensitivity of the antibody used in GlutenTox ELISA Competitive, makes this a method to accurately quantify the gluten content in samples that have undergone enzymatic proteolysis processes (beer, baby food, glucose syrups, etc.) [ref. 4]. Other methods such as the ELISA Sandwich or immunochromatographic sticks could underestimate the amount of the toxic fraction present in hydrolyzed foods [ref. 5].

4. Supplied materials

All reagents supplied are ready to use, except the 10x concentrated Wash Solution stock.

- 12 multi-well coated strips (dividable; 8 wells each)
- 10x Wash Solution (40 mL)
- Dilution Solution (120 mL)
- Extraction Solution (200 ml)
- Substrate Solution (12 mL)
- Stop Solution (0.5 M H_2SO_4)
- GlutenTox Conjugated Reagent (15 ml)
- 5 GlutenTox Standards (1000 to 25 ng/ml hydrolyzed gliadin, 1.25 ml each)
- Negative control (1.25 mL)
- Positive control (1.25 mL)

5. Materials not supplied

- Analytical scale (accurate to 0.1 g)
- Capped centrifuge test tubes (> 10 mL)
- Test vials (1.5-2 mL)
- Disposable gloves
- Distilled water
- Timer
- Vortex mixer
- Tube rotator (or similar mixing device)
- Centrifuge
- Thermostatically-controlled water bath
- Automatic microplate washer (recommended)
- Mono-channel pipettes, multi-channel pipettes (recommended), pipette tips
- ELISA plate reader (with 450 nm filter)

6. Storage conditions and stability

- Store all kit reagents at 2 – 8 °C (36 - 46 °F). Do not freeze.
- Reagents will remain stable until the expiration date, provided they are stored and manipulated correctly.
- Check the expiration date of the components of the kit before starting the test. Do not use any reagent or the multi-well coated strips after the expiration date.
- Unused multi-well strips should be kept in the desiccant-containing aluminum bag, hermetically sealed and stored at 2 – 8 °C (36 – 46 °F).
- Diluted Wash Solution remains stable for two weeks at 2 – 8 °C (36 – 46 °F).

7. Precautions

- Carefully read this manual before performing the assay.
- It is recommended that the instructions described in the manual be followed exactly as described.
- This kit is designed for professional use only.
- Do not mix components from various kits or use reagents or solutions other than those supplied.
- It is recommended that this kit be used with non-powdered disposable gloves. Touching multi-well strips with your hands should be avoided.
- Incomplete sealing of the aluminium bag containing the multi-well strips can result in the accumulation of humidity inside the bag and reduced assay accuracy.

- The Substrate Solution is photosensitive; avoid prolonged light exposure.
- The Stop Solution contains sulphuric acid (H_2SO_4); avoid its ingestion, inhalation, or contact with skin or eyes. Avoid exposure to basic solutions, metals, or other compounds that could react with acids.

8. Recommendations

- Each sample material should be analyzed at least in duplicate.
- Do not use more than 6 multi-well coated strips in one experiment.
- Before the analysis, degassing of carbonated drinks (e.g. beer) is recommended.

General considerations

Samples tested negative could still contain a gluten contamination below the limit of detection of the assay.

WARNING! It is necessary to work carefully and meticulously to obtain exact and reproducible results. A variety of factors are involved in successful assay completion including the initial temperature of the reagents, assay incubation times, precision and reproducibility of liquid handling (pipetting) and quality of the washing technique. Do not allow multi-well coated strips to dry between working steps.

9. Reagent preparation

WARNING! Allow all the reagents to reach room temperature (15 – 25 °C / 59 – 77 °F) before starting the assay.

Preparation of 1x Wash Solution

The Wash Solution is supplied as a 10x concentrate, which must be diluted 1:10 in distilled water prior to use. To dilute all the supplied solution, add the 40 mL of 10x Wash Solution to 360 mL of distilled water. If only part of the Wash Solution is needed at a given time, a smaller quantity can be prepared by following a 1:10 dilution (for example, 60 mL of 1x Wash Solution, enough for a 16- well assay, can be prepared by adding 6 mL of 10x Wash Solution to 54 mL of distilled water). Once diluted, the Wash Solution remains stable for 2 weeks if stored at 2 – 8 °C (36 – 46 °F).

10. Sample preparation

Food samples need to undergo an extraction process in order to make the immunotoxic gluten peptides accessible for subsequent analysis.

The protocol for performing the extraction of the samples depends on the type of food to be analyzed.

NOTE: Once extracted, samples must be analyzed as soon as possible.

10.1. Solid and semisolid samples:

10.1.1. Homogenize, mill and/or triturate the sample.

10.1.2. Weigh 1 g of sample and add it to a test tube.

10.1.3. Add 5 mL of Extraction Solution. Close the tube and mix vigorously using a vortex mixer or similar device.

10.1.4. Depending on the complexity of the sample matrix and whether the food sample has been processed by heat or not, follow one of the 2 options below:

a) Non-heat-processed samples with simple matrix composition
Incubate the sample at room temperature (15-25 °C / 59-77 °F) for 40 minutes with mild agitation (for example, using a tube rotator).

b) Heat-processed sample and/or with complex matrix composition.
Incubate the sample at 50 °C (122 °F) in a water bath for 40 minutes; periodically mix the sample by inverting or vortexing the tube.

NOTE: If the type of sample is difficult to determine, we recommend heating at 50 °C (122 °F), option b, to facilitate the extraction.

10.1.5. Centrifuge the suspension at 2500 x g for 10 minutes and transfer the supernatant to a clean tube.

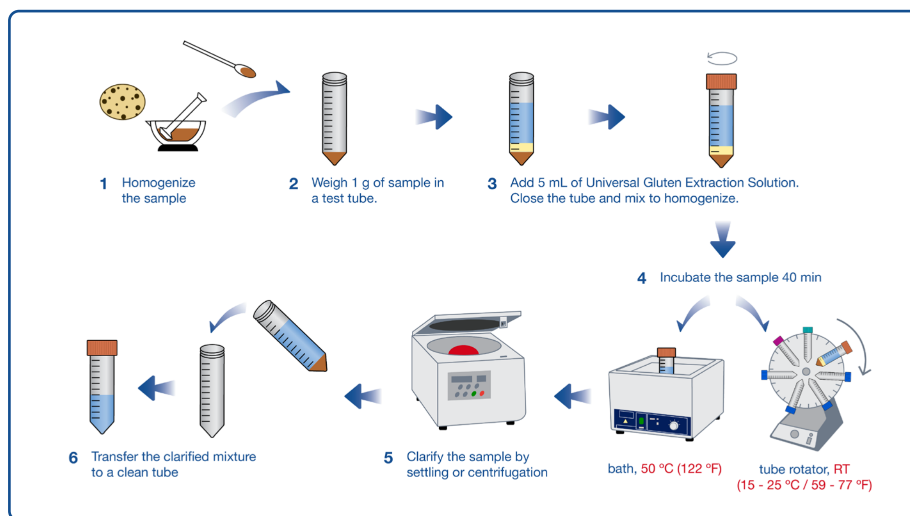


Figure 1. Scheme of the extraction procedure for solid samples

10.2. Liquid samples:

Liquid samples without emulsions or solids do not require intensive extraction. Mild agitation is enough, and the final step of centrifugation is not required.

10.2.1. Shake the sample to homogenize.

10.2.2. Add 1 mL of sample in a test tube.

10.2.3. Add 4 mL of Extraction Solution. Close the tube and shake for 10 minutes using a tube rotator.

10.2.4. If froth is observed, let the sample rest for a few minutes, until the froth has disappeared, before preparing appropriate sample supernatant dilutions.

11. Test procedure:

WARNING! Allow all the reagents to reach room temperature (15 – 25 °C / 59 – 77 °F) before starting the assay.

11.1. All assay reactions (GlutenTox Standards, positive control, negative control and samples) should be performed at least in duplicate. The volumes given below have been calculated using two wells for each reaction.

11.2. For each sample, prepare appropriate sample supernatant dilutions in microcentrifuge tubes using Dilution Solution. A final volume of 300 µL is enough for the analysis of each sample. The recommended sample dilution is 1:10 (30 µL of sample and 270 µL of Dilution Solution). Extracted sample dilutions should be analyzed as soon as possible and any unused material should be discarded.

11.3. Add 100 µL of each standard, positive control, negative control and sample dilution to separate wells, in duplicate (two wells each). Cover the wells and incubate at room temperature (15-25 °C / 59-77 °F) for 30 min.

11.4. Washes: eliminate well contents by inverting the plate; add 300 µL of diluted Wash Solution to all wells; incubate for three seconds. Repeat this sequence four more times, for a total of five washes. Perform the washes in the same order used to load the wells in the previous step. After the last wash, invert the plate and tap it on an absorbent material (for example, a clean paper towel) to eliminate the remaining liquid. An automatic washer is recommended for a higher reproducibility of the results.

11.5. Add 100 µL of the GlutenTox Conjugated Reagent to each well. Cover the wells and incubate at room temperature (15-25 °C / 59-77 ºF) for 30 minutes.

NOTE: GlutenTox Conjugated Reagent should be pipetted following good laboratory practices and in the most aseptic conditions possible. To avoid potential microbial or chemical contamination, never return unused GlutenTox Conjugated Reagent to its original container.

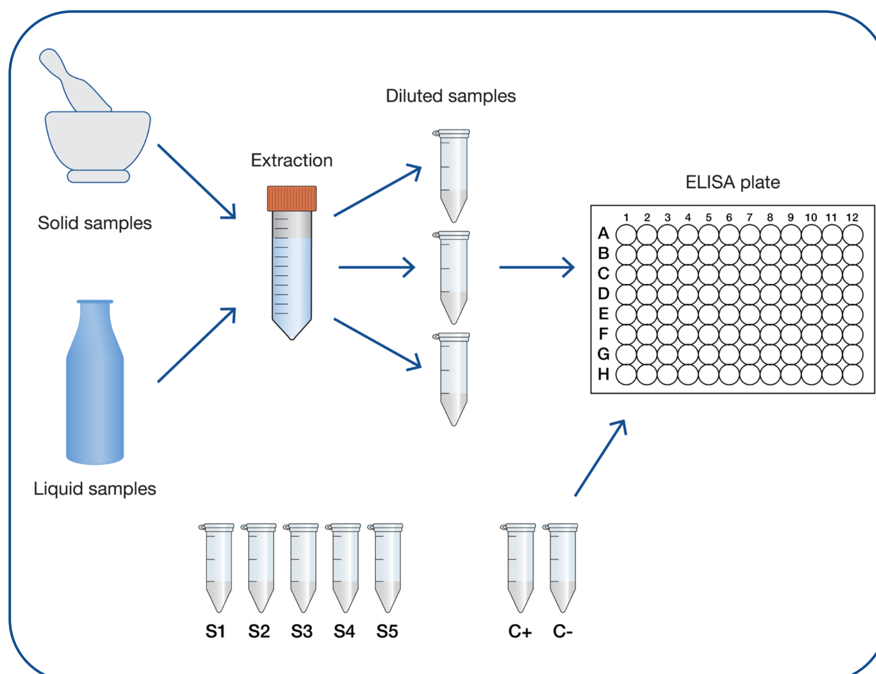


Figure 2. Scheme of the analysis procedure

11.6. Wash the plate five times with 300 µL of Wash Solution per well, as indicated in step 11.4.

11.7. Add 100 µL of Substrate Solution to each well. Cover the wells and incubate at room temperature (15-25 °C / 59-77 °F) for 30 minutes in the dark.

11.8. Add 100 µL of Stop Solution to each well. Follow the same order used when adding the Substrate Solution in the previous step.

11.9. Using an ELISA microplate reader with a 450 nm filter, read the absorbance (OD) of each well as soon as possible, within 30 minutes of the addition of the Stop Solution.

12. Results calculation

12.1. Determine average absorbance values for the duplicates of each condition.

12.2. Using appropriate software (e.g. Excel®), prepare a standard curve (see Fig. 3) by plotting the values of Log (10) [ng/ml of hydrolyzed gliadin] on the Y axis versus the respective absorbance values on the X axis obtained from the GlutenTox Standards. Please contact Hygiena Diagnóstica España to obtain the Excel® template.

12.3. Calculate the equation that defines the standard curve by second-order polynomial regression using suitable software. An example is shown in Figure 3.

12.4. Enter into this equation the sample absorbance values obtained for each sample to obtain the Log (10) value of hydrolyzed gliadin concentrations of the sample dilutions.

12.5. Enter the Log (10) value obtained into the following formula to obtain the hydrolyzed gliadin concentration of the sample dilution.

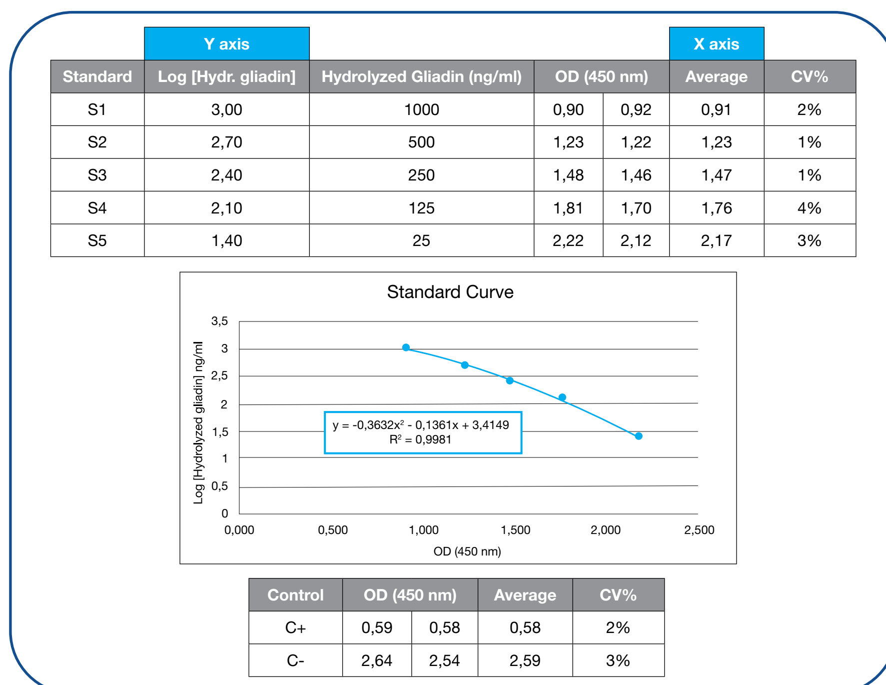
ng/ml hydrolyzed gliadin = 10^(Log(10) [ng/ml of hydrolyzed gliadin])

12.6. Enter the hydrolyzed gliadin concentration value obtained into the following formula to obtain the amount of gluten in ppm.

ppm of gluten = (ng/ml hydrolyzed gliadin x 2 x dilution*)/200

*dilution performed in Step 11.2.

NOTE: When the absorbance (OD) of a sample is not within the values covered by the standard curve, the assay should be repeated using different dilutions.



	Y axis				X axis	
Standard	Log [Hydr. gliadin]	Hydrolyzed Gliadin (ng/ml)	OD (450 nm)		Average	CV%
S1	3,00	1000	0,90	0,92	0,91	2%
S2	2,70	500	1,23	1,22	1,23	1%
S3	2,40	250	1,48	1,46	1,47	1%
S4	2,10	125	1,81	1,70	1,76	4%
S5	1,40	25	2,22	2,12	2,17	3%

Control	OD (450 nm)		Average	CV%
C+	0,59	0,58	0,58	2%
C-	2,64	2,54	2,59	3%

Figure 3. Example of the Standard Curve

13. Quality control

Positive and negative controls are included to ensure that the test has worked correctly. Assay performance can be considered adequate when the absorbance of the positive control is below that obtained from the GlutenTox 1000 ng/ml hydrolyzed gliadin standard (S1) and the absorbance of the negative control is above that obtained from the GlutenTox 25 ng/ml hydrolyzed gliadin standard (S5).

14. Analytical features

Tests have been performed to determine the principal analytical characteristics of the assay.

Sensitivity

The limit of detection (LoD) of the assay is 1.6 ppm gluten/0.8 ppm gliadin. The limit of quantification (LoQ) is 2.5 ppm gluten/1.25 ppm gliadin. The range of quantification of the assay is 2.5-100 ppm gluten (1.25-50 ppm gliadin) using 1:10 sample dilution.

Specificity

This test can specifically detect the presence of the toxic fraction of the prolamins of wheat (gliadin), rye (secalin), barley (hordein) and some varieties of immunogenic oats (avenin) that can therefore be harmful for celiac patients [ref. 2]. However, when the samples contain celiac-safe foods like rice, corn, soy, buckwheat, sesame, millet, teff, quinoa and amaranth, no positive signal is observed.

15. Intellectual Property

The immunoreagents used in this kit are commercialized under the exclusive license for biological material from the Spanish National Research Council (CSIC).

16. References

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4. DENERY-PAPINI S., *et al.*; "Extraction and immunochemical measurement of raw and heated gluten in food", 15th Meeting Working Group on Prolamin analysis and toxicity, Nov 2000, Meran, Italy. pp. 139-142.

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Notes

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