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# GlutenTox® ELISA Competitive G12

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

REF KIT3012 (KT-4758)

# GlutenTox® ELISA
## Competitive G12

# Contents

## 1. Intended Purpose

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 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]. The G12 antibody is conjugated to HRP (horseradish peroxidase), which, in the presence of a substrate, produces a detectable signal. Subsequently, the sample-antibody mixture is added to a multi-well plate coated with gliadin. The competitive ELISA is an indirect method, where the lower the quantity of gluten present in the sample, the more antibody remains free to bind to the gliadin coated on the well, resulting in a stronger HRP-signal.

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

- 12 multi-well gliadin-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, 1M $H_2SO_4$ (12 mL)
- GlutenTox G12-HRP conjugated antibody (15 mL)
- GlutenTox Standard Stock (desiccated) (4x)

All supplied reagents are ready to use, except the desiccated GlutenTox standard Stocks and the Wash Solution that has a concentration of 10x.

## 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 (optional)
- Mono-channel pipettes, multi-channel pipettes (recommended), pipette tips

For testing food containing polyphenols (including tannins) and cosmetics containing antioxidants, please acquire the Polyphenol Pack (KT-5320/KIT3008)*, available from Hygiena™. This pack contains:

- Special polyphenol additive (25 g).
- Positive Control containing polyphenols (cocoa powder with gluten, 10 g).
- Negative Control containing polyphenols (gluten-free cocoa powder, 10 g).

**IMPORTANT NOTE!: Foods rich in polyphenols or tannins are: chocolate, tea, coffee, wine, purple corn and corn fiber, soy, berries, etc.**

**IMPORTANT NOTE!: The most common antioxidants in cosmetic products are vitamins A, C and E, carotenes, carotenoids, etc.**

* For more information contact your supplier.

## 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 plate after the expiration date.
- Unused multi-well strips should be hermetically sealed in the desiccant-containing aluminum bag and stored at 2 – 8 °C (36 - 46 °F).
- Diluted Wash Solution remains stable for two weeks at 2 – 8 °C (36 – 46 °F).
- Resuspended GlutenTox Standard Stocks can be stored at 2 - 8 ºC (36 - 46 ºF) for a maximum of 24h.

## 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 aluminum 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.

**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 reagents, assay incubation times, precision and reproducibility of liquid handling (pipetting) and quality of the washing technique.

# 8. Reagent preparation

### 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 of 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 following a 1:10 dilution (for example, 60 mL of 1x Wash Solution, sufficient 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).

### Preparation of the Standards

Take one of the four supplied GlutenTox Standard Stocks (*the desiccated material is transparent, and you may not see it*). Add 250 µl ethanol 60% v/v to the tube. Let it stand at room temperature for 5-10 min to rehydrate. Longer incubation times do not affect the assay results (*you can use this time to prepare the rest of the material (label the tubes, dilute the extracted samples, etc.*). Mix the content of the tube by pipetting or using a vortex mixer to ensure a total resuspension of the desiccated material.

> **NOTE!: Pipetting ethanol requires the utmost attention so that the volume aspirated and dispensed is as accurate as possible.**

Add 100 µL of the resuspended standard into a tube with 900 µL of Dilution Solution to get a final concentration of 400 ppm gluten. This is your positive control.

The quantification standards are made by performing six serial dilutions 1:2 from the positive control (C+, 400 ppm gluten) to Standard 6 (S6, 6.25 ppm gluten). (See Figure 1 and Annex 1.1 for detailed instructions). The upper limit of quantification is Standard 1 (200 ppm gluten). The negative control corresponds to Dilution Solution.

> **NOTE!: Prepare the Standards just before preparing the Competition Mixtures. Once the standards S1 to S6 have been prepared, they must be used as soon as possible. Do not store them.**

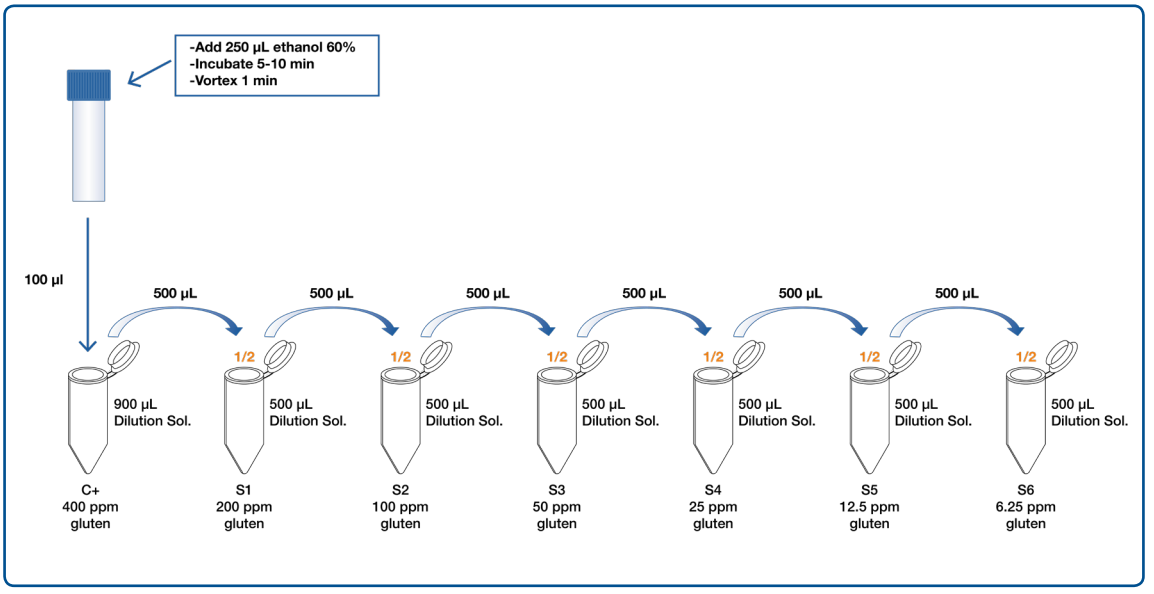


Figure 1. Scheme for the preparation of GlutenTox Standards.

# 9. Sample extraction

Food samples need to undergo an extraction process in order to make the toxic 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.**

## 9.1. Solid and semisolid samples

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

9.1.2. Weigh 0.5 g of sample and add them to a test tube.

**NOTE!: If the sample, whether solid or liquid, contains polyphenols, tannins or antioxidants, weigh and add to the tube containing the sample 0.5 g of the special additive for polyphenols. (See Annex 1.3 for a detailed protocol).**

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

9.1.4. 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 ºC / 59 - 77 ºF) for 40 minutes with mild agitation (for example, using a tube rotator).

**b) Heat-processed samples and/or with complex matrix composition, or samples containing polyphenols, tannins or antioxidants.**
Incubate the sample at 50 ºC (122 ºF) in a water bath for 40 minutes and 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 ºC (122 ºF) (option b) to facilitate the extraction.**

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

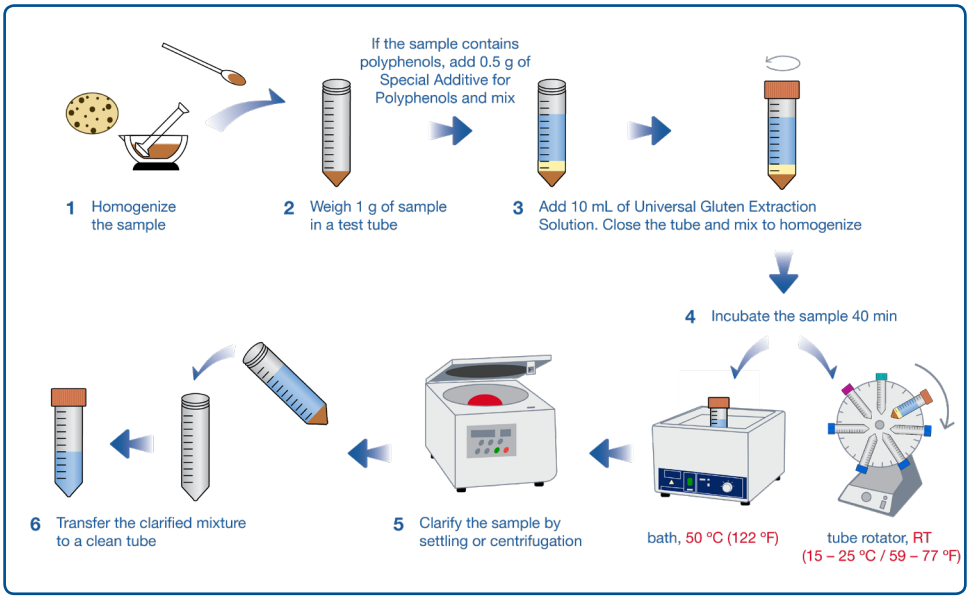


Figure 2. Scheme of the extraction procedure for solid samples.

### 9.2. Liquid samples

**NOTE!: Liquid samples with polyphenols, tannins or antioxidants have to be analyzed according to point 9.1. Solid and semisolid samples.**

Liquid samples without emulsions or solids do not require intensive extraction, therefore 1 or 2 minutes of manual shaking is sufficient, and the final step of centrifugation is not required.

9.2.1. Shake the sample to homogenize.

9.2.2. Add 0.5 mL of sample in a test tube.

9.2.3. Add 4.5 mL of Extraction Solution. Close the tube and shake 1-2 minutes manually or using a vortex mixer.

## 10. Sample analysis

It is recommended that all assay conditions (GlutenTox Standards, positive control, negative control, and samples) be analyzed in duplicate as a minimum. For this reason, the volumes given below have been calculated using two wells for each sample.

**10.1.** Allow all the reagents to reach room temperature (15 – 25 °C /59 – 77 °F) before starting the assay, with the exception of the GlutenTox G12-HRP conjugated antibody, which should be kept at 2 – 8 °C (36 – 46 °F) until use.

**10.2.** For each sample, prepare appropriate sample supernatant dilutions in microcentrifuge tubes using Dilution Solution. A final volume of 300 µL is needed for the analysis of each sample. The recommended sample dilution is 1:10 (30 µL of sample and 270 µL of Dilution Solution). Sample dilution should be analyzed as soon as possible.

**10.3.** Prepare the Competition Mixtures: add 300 µL of GlutenTox G12-HRP conjugated antibody to each tube with the 300 µL of diluted sample. In fresh microcentrifuge tubes, mix 300 µL of the 6 GlutenTox Standards, positive and negative controls with 300 µL of G12-HRP conjugated antibody. Incubate the assay mixtures (sample-antibody, Standards-antibody and controls-antibody) for 60 minutes at room temperature (15 – 25 °C /59 – 77 °F) with mild agitation (preferably using a tube rotator).

**NOTE!: GlutenTox G12-HRP conjugated antibody 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 G12-HRP conjugated antibody to its original container.**

**10.4.** Put the required number of multi-well strips into the support. Immediately return unused strips to the aluminum bag and seal firmly to minimize humidity exposure.

**10.5.** Plate loading: add 200 µL of the Competition Mixtures (sample-antibody, standards-antibody and controls-antibody) to each well (two per mixture). This procedure should be done **as fast as possible** (see Annex 1 for a detailed protocol). Cover the wells and incubate at room temperature (15 – 25 °C /59 – 77 °F) for 60 minutes.

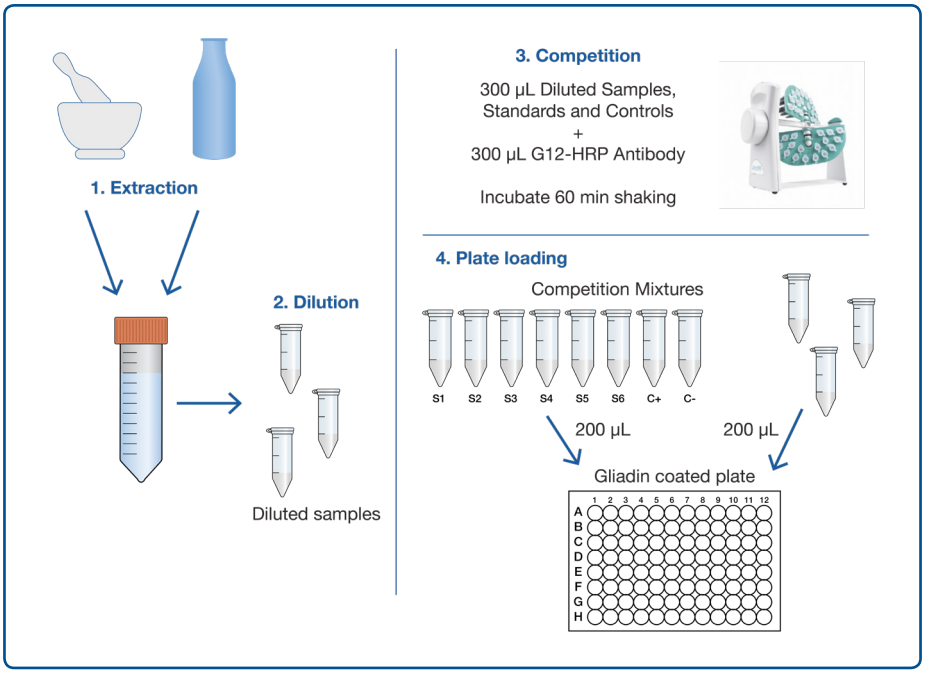


Figure 3. Scheme of the analysis procedure.

**10.6.** Washes: eliminate wells content by inverting the plate; add 300 µL of diluted Wash Solution to all wells; incubate a few seconds. Repeat this sequence four more times, for a total of five washes. Perform the washes **in the same order as Competition Mixtures were prepared and loaded**. After the last wash, invert the plate and tap it on an absorbent material to eliminate the remaining liquid.

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

**10.8.** Add 100 µL of Stop Solution to each well. Follow the same order as Substrate Solution was added.

**10.9.** Read the absorbance (OD) at 450 nm of each well within an hour of the addition of the Stop Solution.

## 11. Results calculation

**11.1.** Determine average absorbance values for the duplicates of each condition.

**11.2.** Using appropriate software (e.g. Excel), prepare a standard curve (see Fig. 4) by plotting the values of log [ppm of gluten] 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.

**11.3.** Calculate the equation that defines the standard curve by second-order polynomial regression using suitable software. An example is shown in Figure 4.

**11.4.** Enter into this equation the sample absorbance values to obtain the log value of gluten concentrations. To obtain the ppm value use the following formula:

$$\text{ppm of Gluten} = 10^{\log[\,]}$$

**11.5.** Please note that the 1:10 dilution factor of the samples is already included in the calculations. If you used a dilution factor greater than 1:10, you will need to multiply your result by this factor as follows: a dilution factor of 1:50 means you need to multiply by 5, a dilution factor of 1:100 means you need to multiply by 10, etc.

**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.**

| Standard | ppm gluten | log [ ] | ABS 1 | ABS 2 | Average | CV% |
|---|---|---|---|---|---|---|
| S1 | 200 | 2.30 | 0.95 | 0.94 | 0.95 | 0% |
| S2 | 100 | 2.00 | 1.13 | 1.06 | 1.10 | 4% |
| S3 | 50 | 1.70 | 1.23 | 1.25 | 1.24 | 1% |
| S4 | 25 | 1.40 | 1.38 | 1.38 | 1.38 | 0% |
| S5 | 12.5 | 1.10 | 1.47 | 1.46 | 1.47 | 1% |
| S6 | 6.25 | 0.80 | 1.60 | 1.69 | 1.64 | 4% |
| C+ | | | 0.94 | 0.89 | 0.92 | 4% |
| C- | | | 1.73 | 1.84 | 1.78 | 4% |

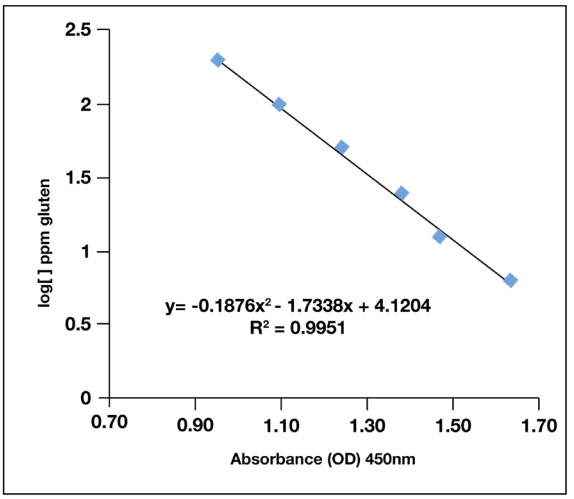


Figure 4. Example of the Standard Curve

## 12. Quality control

An internal positive control is 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 200 ppm standard (S1) and the absorbance of the negative control is above that obtained from the GlutenTox 6.25 ppm standard (S6).

## 13. Analytical features

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

### Sensitivity

The range of quantification of the assay is 6.25-200 ppm gluten.

### 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.

## 14. Intellectual property

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

## 15. References

1. SHAN L., *et al.*; "Structural basis for gluten intolerance in celiac sprue"; Science; 2002; 297: 2275-9.

2. COMINO I., *et al.*; “Diversity in oat potential immunogenicity: basis for the selection of oat varieties with no toxicity in coeliac disease.”; Gut; 2011; 60:915-922.

3. MORÓN B., *et al.*; “Toward the Assessment of Food Toxicity for Celiac Patients: Characterization of Monoclonal Antibodies to a Main Immunogenic Gluten Peptide” PLoS ONE 2008; 3(5): e2294.

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.

5. DOSTÁLEK, *et al.*; "Food additives & contaminants", 2006; 23: 1074-1078.

# ANNEX 1. Recommended protocols

## 1. Preparation of the GlutenTox Standards

Preparation of the calibration curve with the GlutenTox Standards is an essential part of the GlutenTox Competitive G12 assay. Here we describe in detail the procedure to prepare these standards in order to achieve accurate and reproducible results.

1.1. Add 250 µL ethanol 60% v/v to one of the four supplied GlutenTox Standards with desiccated material.

1.2. Let it stand at room temperature (15 – 25 °C /59 – 77 °F) for 5-10 minutes. Longer incubation times do not affect assay results.

1.3. Prepare the microcentrifuge vials you will need for the assay (see Fig. 1): 8 for the GlutenTox Standards, 8 for the Competition Mixtures of the Standards, plus 1 vial per sample to prepare the sample dilution and the Competition Mixture. Label them and add Dilution Solution as needed.

1.4. Vortex the GlutenTox Standard tube for 1 min until complete resuspension of the desiccated material.

1.5. Add 100 µL from the resuspended material to the C+ tube containing 900 µL Dilution Solution. Pipette the mixture up and down 6 times. Vortex the tube for 5 seconds.

1.6. Serial dilutions: with a fresh pipette tip, take 500 µL from the C+, wet the tip by pipetting up and down 3 times and dispense them in the S1 tube containing 500 µL of Dilution Solution by pipetting up and down five times. Repeat the same procedure to transfer the solution to the next standard tube (3 times before transferring, 5 times for washing).

1.7. Repeat this procedure for the rest of the Standards (S2, S3, S4, S5 and S6). Use the same pipette tip to perform all serial dilutions.

**WARNING!** The more consistent the procedure of preparation of the Standards, the more accurate and reproducible the results will be. Wetting the tip and pipetting up and down in the same way in all the standards ensures that you are transferring the exact amount of liquid from one tube to the next.

## 2. Preparation of the Sample Dilutions, Competition Mixtures and plate loading

Competition of extracted samples/G12-HRP antibody and GlutenTox Standard/G12-HRP antibody is the core of the competitive assay. **It is important that these mixtures are prepared carefully.**

2.1. Label all the needed microcentrifuge vials.

2.2. Prepare the sample dilutions (30 µL sample and 270 µL Dilution Solution) in the tubes for the Samples/Antibody mixtures.

2.3. Dispense 300 µL of each Standard, positive and negative controls in the vials for the Standard/Antibody mixtures.

2.4. Working as fast as possible, dispense 300 µL of G12-HRP conjugated antibody in each of the vials prepared in 2.2 and 2.3, and close them tightly.

2.5. Incubate the tubes exactly 60 min at room temperature (15 – 25 °C /59 – 77 °F) with mild agitation, preferably in a tube rotator.

2.6. Put the vials in a rack in the same order as they will be loaded in the gliadin-coated plate.

2.7. Working as fast as possible, dispense 200 µL of all the Competition Mixtures in the corresponding well in duplicate (two wells per condition). When aspirating the volume from the original tube, wet the pipette tip by pipetting up and down two times before dispensing the volume in the well. Loading a whole plate (96 wells) should not take more than 10 minutes. **Longer loading times may affect the assay results.**

## 3. Extraction procedure for food containing polyphenols, tannins or antioxidants

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

3.2. Weigh 0.5 g or add 0.5 mL of sample in a test tube.

3.3. Add 0.5 g of the additive for polyphenols. Mix vigorously using a vortex mixer until the two kinds of powders or the powder and the liquid form a homogeneous mixture.

3.4. Add 5 mL of Extraction Solution.

3.5. Mix vigorously using a vortex mixer until complete disaggregation. With some samples it can be helpful to pre-heat the sample a couple of minutes at 50 ºC (122 ºF) and then vortex again until complete disaggregation.

3.6. Once completely disaggregated, use option 9.1.4 b) for incubation (40 minutes at 50 ºC (122 ºF)) and follow the rest of the procedure as usual.

GlutenTox® ELISA
Competitive G12

## Notes

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