hygiena™

GlutenTox® ELISA Sandwich

Kit for gluten determination in foodstuff

KEY diagnostics
T: 02 8212 4074 F: 02 9423 6992
info@keydiagnostics.com.au
www.keydiagnostics.com.au
PO Box 1038, Gymea, NSW, 2227

REF KIT3011

GlutenTox® ELISA Sandwich

Kit for gluten determination in foodstuff

Contents

1. Intended use

GlutenTox® ELISA Sandwich is an enzyme-linked immunosorbent assay (ELISA) for the determination of gluten*, which is harmful for celiac disease sufferers, in food and beverages.

* Not for hydrolyzed sources of gluten.

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 (mg/kg).

3. Test basis

GlutenTox ELISA Sandwich is a quantitative enzyme-linked immunosorbent assay (ELISA) designed for the detection and quantification of the immunotoxic fraction of 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 sample’s extract is added to a multi-well plate coated with monoclonal anti-gliadin antibody (A1) that specifically recognize the most toxic or immunogenic fraction of gluten. After the washing steps, the addition of a second monoclonal antibody conjugated to HRP (G12-HRP) will allow to measure the signal. GlutenTox ELISA Sandwich is a direct method. The higher the concentration of gluten present in the sample, the more intense the signal will be.

The ELISA Sandwich is a usual technique for the analysis of substances found at very low concentrations. The high specificity of the antibodies used in this test [ref. 3], allows this method to precisely quantify gluten in food samples.

4. Supplied materials

- 12 multi-well A1-coated strips (dividable; 8 wells each)
- Wash Solution 10x (40 mL)
- Dilution Solution (120 mL)
- Extraction Solution (200 mL)
- GlutenTox G12-HRP conjugated antibody (15 mL)
- Substrate Solution (12 mL)
- Stop Solution, H_2SO_4 1M (12 mL)
- GlutenTox Standard Stock (desiccated) (4x)

All supplied reagents are ready to use, except the desiccated GlutenTox Standard Stocks and the 10x concentrated Wash Solution stock.

5. Materials not supplied

- Analytical scale (accurate to 0.1 g)
- Capped centrifuge tubes (> 10 mL)
- Test polypropylene vials (1.5-2 mL)
- ELISA Microplate reader (with 450 nm filter)
- 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, multichannel pipettes (recommended) and disposable pipette tips
- Ethanol 60% v/v

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

- Special polyphenol additive (ASY3044) (25 g).
- Positive control containing polyphenols (ASY3043) (cocoa powder with gluten, 10 g).
- Negative control containing polyphenols (ASY3042) (gluten-free cocoa powder, 10 g).

NOTE: Foods rich in polyphenols or tannins are: chocolate, tea, coffee, wine, purple corn and corn fiber, soy, berries, legumes like chickpeas or lentils, etc.

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 ºF). 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 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 ºF).
- Diluted Wash Solution remains stable for two weeks at 2-8 °C (36-46 ºF).
- Resuspended GlutenTox Standard Stock can be stored at 2-8 °C (36-46 ºF) for a maximum of 24 hours.

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 powder-free 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.**

8. Recommendations

- **Each sample material should be analyzed at least in duplicate.**
- **Use gluten-free and gluten-containing (spiked) samples as test controls.**
- **Due to the high variability of food types, matrix effects cannot be excluded. To ensure an accurate result, the analysis of spiked samples is recommended.**
- **In the production of foods such as beer or sourdough, gluten proteins are fragmented. In sandwich ELISAs protein fragments lead to a reduced recovery. Such samples should be analyzed with a competitive ELISA test system.**

General considerations

- Samples tested negative could still contain a gluten contamination below the limit of detection of the assay.
- Due to the high variability of food types, matrix effects cannot be excluded. In processed food (e.g. heat treatment, dehydration, etc.) proteins may be altered or fragmented; this may have an impact on the recovery/cross reactivity.

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.

9. Reagent preparation

WARNING! Allow all the reagents to reach room temperature (15-25 °C / 59-77 ºF) before starting the assay, except for GlutenTox G12-HRP conjugated antibody, which should be kept at 2-8 ºC (36-46 ºF) until use.

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, 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 ºC (36-46 ºF).

Preparation of Standards

Take one of the four supplied GlutenTox Standard Stocks. (*The material is transparent, and you may not see it.*) Add 250 µL of ethanol 60% v/v into 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 for 1 minute 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 75 µL of the resuspended standard into a tube with 825 µL of Dilution Solution to get a final concentration of 100 ng/mL of gliadin. This is your positive control.

The quantification standards are made by performing six serial dilutions 1:2 from the positive control (C+, 100 ng/mL of gliadin) to Standard 6 (S6, 1.56 ng/mL of gliadin). (See Figure 1). The upper limit of quantification is Standard 1 (S1, 50 ng/mL). The negative control corresponds with Dilution Solution. See a detailed protocol in Annex 1.1.

> **NOTE: Prepare the standards just before adding them to the coated plate. Once the standards S1 to S6 have been prepared, they must be used as soon as possible. Do not store them.**

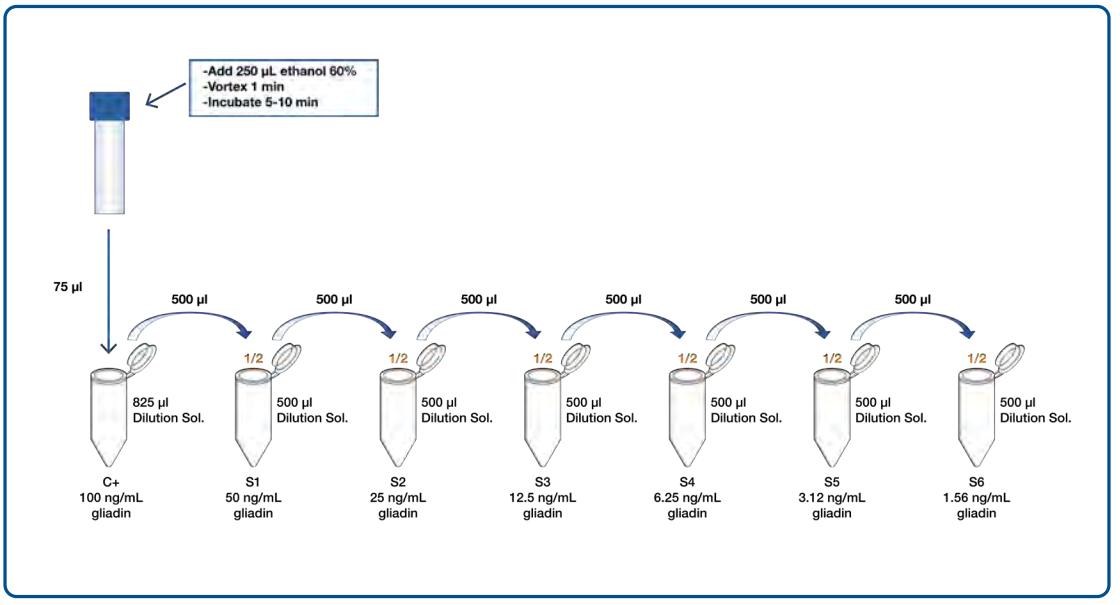

Figure 1. Scheme for the preparation of GlutenTox Standards.

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 0.5 g of sample in 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 (KIT3008) and shake vigorously to mix. (See Annex 1.2 for a detailed protocol).

10.1.3. Add 5 mL of Extraction Solution. Close the tube and mix vigorously using a vortex 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 ºC / 59-77 ºF) for 40 minutes with mild agitation (for example, using a tube rotator).

b) Heat-processed sample and/or with complex matrix composition, or samples containing polyphenols, tannins or antioxidants (Annex 1.2).

Incubate the sample at 50 °C (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 ºC) (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.

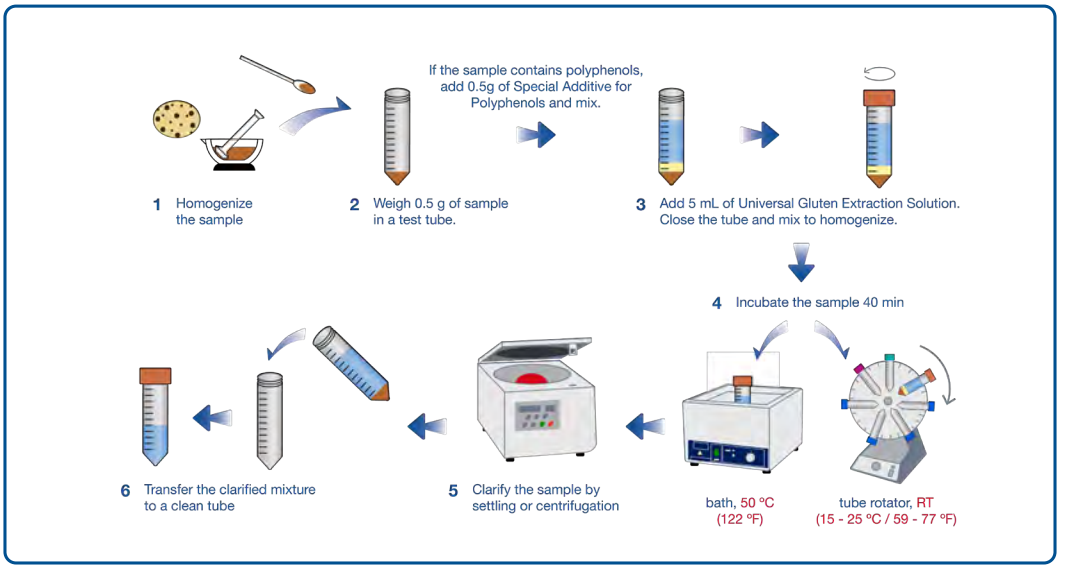

Figure 2. Scheme of the extraction procedure for solid samples.

10.2. Liquid samples

NOTE: Liquid samples with polyphenols, tannins or antioxidants must be analyzed according to the point 10.1. Solid and semisolid samples.

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

10.2.1. Shake the sample to homogenize.

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

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

11. Test procedure

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. Prepare appropriate dilutions of the extracted, clarified samples, using the provided Dilution Solution, and polypropylene vials. A final volume of 300 µL is enough for the analysis of each sample. Extracted sample dilutions should be analyzed as soon as possible, and any unused material should be discarded.

Depending on the expected gluten content of the sample, prepare dilutions according to the following table:

		Example of dilution	
Expected amount of gluten	Dilution	Extracted sample	Dilution Solution
Gluten-free (<20 ppm)	1:50	20 µL	980 µL
Low gluten (20 to 100 ppm)	1:200	10 µL	1990 µL
High gluten (100 to 200 ppm)	1:400	5 µL	1995 µL

11.3. Add 100 µL of each GlutenTox 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 60 min.

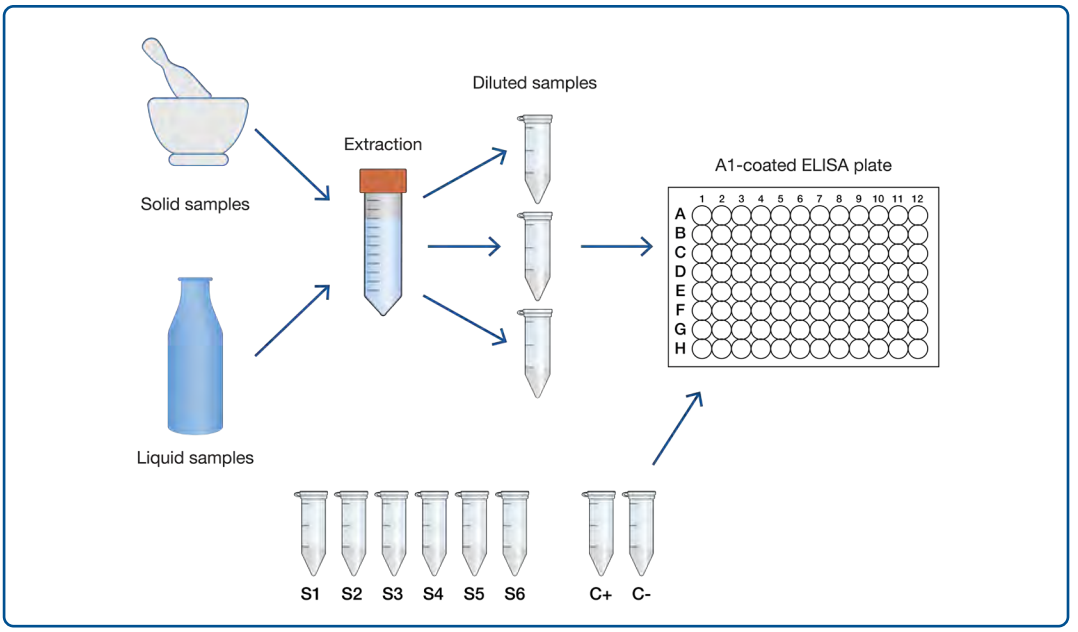

Figure 3. Scheme of the analysis procedure.

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 G12-HRP conjugated antibody to each well. Cover the wells and incubate at room temperature (15-25 °C / 59-77 ºF) for 60 minutes.

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.

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 ºF) 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. Prepare a standard curve (see Figure 4) by plotting gliadin concentrations of each GlutenTox Standard (y-axis) versus the respective absorbance values (x-axis) obtained from the calibration standards using an appropriate software (for example Excel). 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 a suitable software (for instance Excel). An example is shown in Figure 4.

	Y axis			X axis	
Standard	Gliadin (ng/mL)	OD (450nm)		Average	CV%
S1	50	1.50	1.38	1.44	6%
S2	25	0.97	1.02	0.99	3%
S3	12.5	0.67	0.65	0.66	2%
S4	6.25	0.37	0.38	0.37	1%
S5	3.12	0.23	0.27	0.25	10%
S6	1.56	0.17	0.17	0.17	1%
	C+	1.94	1.95	1.95	0%
	C-	0.10	0.11	0.10	9%

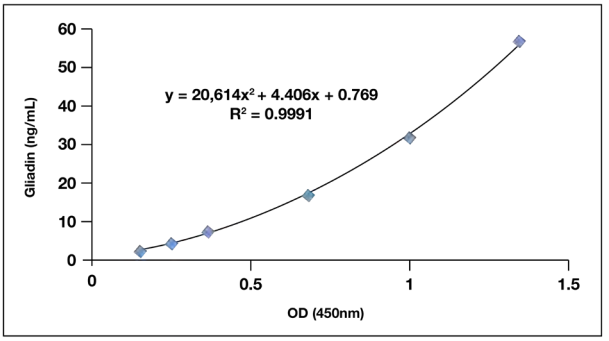

Figure 4. Example of the Standard Curve

12.4. Enter into this equation the sample absorbance values obtained for each sample to obtain gliadin concentrations of the sample dilutions.

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

ppm gluten= (ng/mL gliadin x dilution * x2) /100

*dilution performed in step 11.2

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

13. Quality control

An internal quality 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 above that obtained for the Standard 1 (S1, 50 ng/mL of gliadin) and the absorbance of the negative control is below that obtained for the Standard 6 (S6, 1.56 ng/mL of gliadin).

If this control fails, the assay should be repeated.

14. Analytical features

Tests have been performed to determine the main analytical characteristics of the assay:

Sensitivity

The limit of detection (LoD) is 0.3 ppm gluten/0.15 ppm gliadin. The limit of quantification (LoQ) is 1.6 ppm gluten/0.8 ppm gliadin. The range of quantification of the assay is 1.6-800 ppm gluten (0.8-400 ppm gliadin). Depending on the sample dilution analyzed, the quantifiable quantity of gluten in each sample will vary within the range of absorbance values of the standard curve (see example in the following table):

Sample dilution	Lower quantification limit (ppm gluten)	Upper quantification limit (ppm gluten)
1:50	1.6	100
1:100	3.1	200
1:200	6.2	400
1:400	12.5	800

Specificity

This test is based on the A1 and G12 monoclonal antibodies which can specifically detect the presence of the immunotoxic 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 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

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.

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 ELISA Sandwich 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 test (see Fig. 1): 7 for the GlutenTox Standards and positive control, plus 1 vial per sample to prepare the sample dilution. 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 75 µL from the resuspended material to the positive control C+ tube containing 825 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 positive control 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 before transferring, 5 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. Extraction procedure for foods and drinks containing polyphenols, tannins or antioxidants

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

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

2.3. 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.

2.4. Add 5 mL of Extraction Solution.

2.5. 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 °C (122 ºF) and then vortex again until complete disaggregation.

2.6. Once completely disaggregated, use option 9.1.4 b) for incubation [40 minutes at 50 °C (122 ºF)] and follow the rest of the procedure as usual.

Notes

Notes

Notes

Americas:

Hygiena Headquarters
941 Avenida Acaso
Camarillo, CA 93012
1-805-388-8007

Hygiena Canada
2650 Meadowvale Blvd Unit 14
Mississauga, Ontario L5N 6M5
1-833-494-4362 (Toll-free)
or 1-416-686-7962

Hygiena Mexico, S.A. de C.V.
Calle 3 Anegas 409 Bodega 5, Col. Nueva Industrial
Vallejo, Delegación Gustavo A. Madero, C.P. 07700,
CDMX, México.
+52 (55) 5281-4108 y 5281-4146

International:

Hygiena International
8, Woodshots Meadow
Watford, Hertfordshire
WD18 8YU, UK
+44 (0)1923-818821

Hygiena (Shanghai) Trading Co., Ltd.
Rm.7K, No.518, Shangcheng Rd.
Pudong New District
Shanghai, China 200120
+86 21-5132-1081, +86 21-5132-1077,
+86 21-5132-1078

Hygiena Diagnóstica España S.L.
P. I. Parque Plata, Calle Cañada Real 31-35,
41900, Camas, Sevilla, Spain
+34 954-08-1276

www.hygiena.com

enquiries@hygiena.com

INS3006 REV A