GlutenTox® ELISA

RAPID G12

Kit for gluten determination in foodstuff

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GlutenTox® ELISA Rapid G12

Kit for gluten determination in foodstuff

Contents

1. Intended use

GlutenTox ELISA Rapid G12 is an enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of gluten (from wheat, barley, rye and oat)* which is harmful for celiac disease sufferers, in food (unprocessed or processed)** and beverages.

* Not for hydrolyzed sources of gluten.

** Matrices validated according to AOAC *Performance Tested Methods* (*PTM*) protocols:
Food Matrices: Soy flour, corn bread, seasoning mix, rolled oats, evaporated milk and incurred bread matrix.

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 Rapid G12 is a quantitative enzyme-linked immunosorbent assay (ELISA) designed for the determination of the immunotoxic fraction of gluten in food samples (unprocessed or processed).
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 a monoclonal anti-gliadin antibody (G12) that specifically recognizes the most toxic or immunogenic fraction of gluten. After the washing steps, the addition of a second monoclonal anti-gliadin antibody conjugated to HRP (A1-HRP) and the substrate solution (TMB) will allow to measure the signal (color change). GlutenTox ELISA Rapid G12 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 G12-coated strips (dividable; 8 wells each)
- Wash Solution 10x (100 mL)
- Dilution Solution (60 mL)
- Extraction Solution (200 mL)
- GlutenTox A1-HRP conjugated antibody (15 mL)
- Substrate Solution (12 mL)
- Stop Solution, H_2SO_4, 0.5 M (12 mL)
- 6 GlutenTox Standards (0 to 50 ng/ml gliadin, 1.25 mL each)
- Positive control (1.25 mL)
- Internal control (1.25 mL)

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

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)
- Polyphenol Pack (KIT3008)

For testing **food containing polyphenols (including tannins) and cosmetics 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.

* For more information, contact your supplier.

6. Storage conditions and stability

- Store all kit reagents at 2 to 8 °C (36 to 46 °F). Do not freeze.
- Shelf life of the kit is 12 months.
- 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 G12-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 to 8 °C (36 to 46 °F).
- Diluted Wash Solution remains stable for two weeks at 2 to 8 °C (36 to 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 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 acid.**

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 testing negative could still contain gluten contamination below the limit of detection of the assay (0.4 ppm gluten).
- 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.
- For the evaluation of the cross-reactivity, only one exemplary sample of each matrix was analyzed. All cross-reactivities and exemplary matrices are described in the internal validation report. Other samples may show a different result.

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 to 25 °C / 59 to 77 °F) before starting the assay, except for GlutenTox A1-HRP conjugated antibody, which should be kept at 2 to 8 °C (36 to 46 °F) 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, 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 to 8 °C (36 to 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. Take a representative sample of the food and mill and/or triturate it thoroughly.

10.1.2. Homogenize by shaking the sample by hand for 1 minute. Then weigh 0.5 g of the sample into 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 Appendix 1 A for a detailed protocol).

10.1.3. Add 5 mL of Extraction Solution. Close the tube and mix vigorously using a vortex mixer or similar device for 30 seconds.

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 to 25 °C / 59 to 77 °F) 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 (Appendix 1 A)
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.

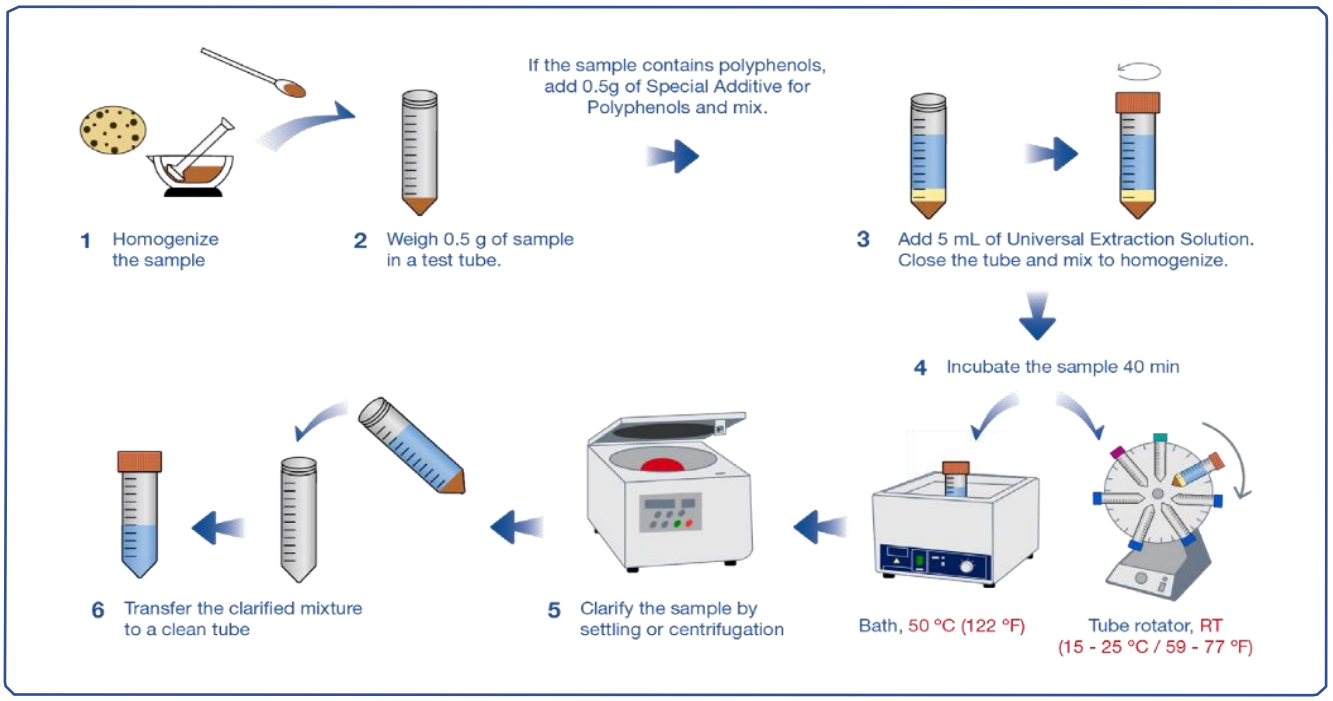

Figure 1. Scheme of the extraction procedure for solid samples

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

10.2. Liquid samples:

NOTE: Liquid samples with polyphenols, tannins or antioxidants have to be analyzed according to the steps in section 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 the 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.

10.3. Swab samples:

NOTE: This procedure can only be used for a qualitative detection of gluten (absence/ presence) in a surface.

Swab sample results should not be used for quantification. For surface sample collection, wet the swab by dipping it in a vial with Dilution Solution. Swab firmly and thoroughly a square of 16 cm^2/2.46 $inch^2$ of the selected surface (see Figure 2). Cut the tip of the swab and put it in a 1.5 mL vial, add 1mL of Dilution Solution and vortex for 1 minute.

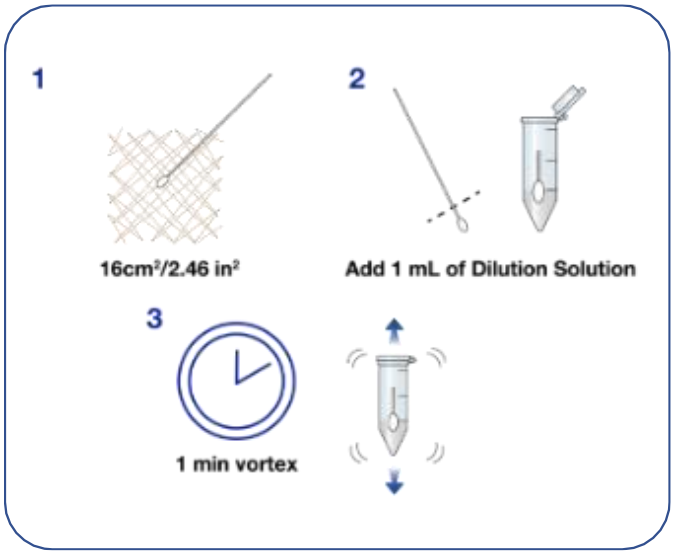

Figure 2. Scheme of the swab sample preparation

Follow the instructions from point 11.3 in the Test procedure section.

11. Test procedure

11.1. All assay reactions (GlutenTox Standards, positive control, internal 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**	**Sample extract**	**Dilution Solution**
Gluten-free (<20 ppm)	1:20	50 µL	950 µL
Low gluten (20 to 50 ppm)	1:50	20 µL	980 µL
Medium gluten (50 to 100 ppm)	1:100	10 µL	990 µL
High gluten (100 to 200 ppm)	1:200	5 µL	995 µL

11.3. Add 100 µL of each standard, positive control, internal 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 A1-HRP conjugated antibody to each well. Cover the wells and incubate at room temperature (15 to 25 °C / 59 to 77 °F) for 30 minutes.

NOTE: GlutenTox A1-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 A1-HRP conjugated antibody to its original container.

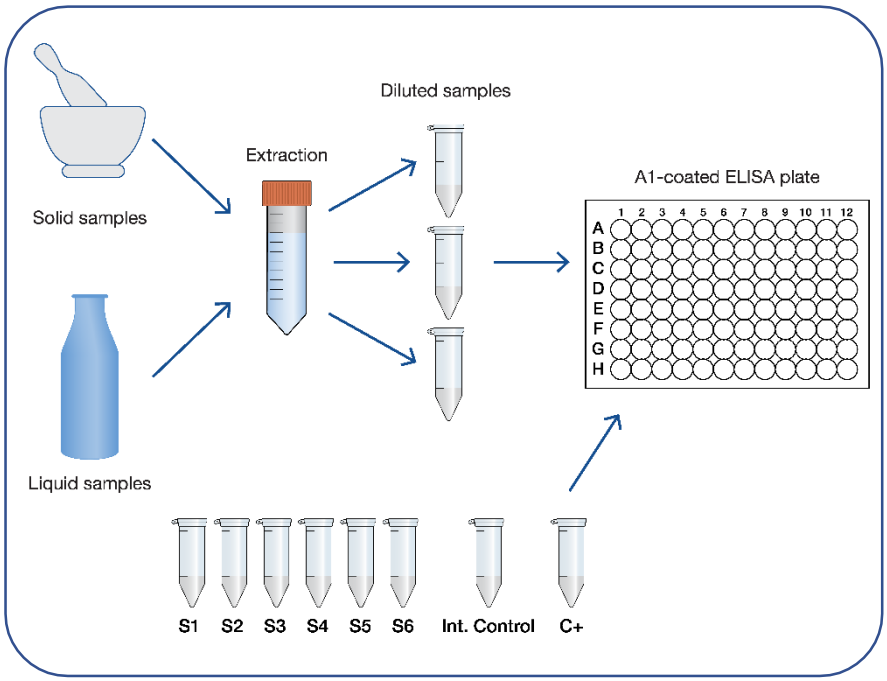

Figure 3. Scheme of the analysis procedure

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 to 25 °C / 59 to 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 replicates 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 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.

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* x 2)/100

**dilution performed on 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.

3. Standard Curve					
Standard	ng/mL Gliadin	OD1	OD2	Average	C.V.%
Std. 1	50	2,109	2,084	2,097	0,8
Std. 2	25	1,205	1,145	1,175	3,6
Std. 3	12,5	0,656	0,636	0,646	2,2
Std. 4	6,25	0,354	0,366	0,360	2,4
Std. 5	1,56	0,138	0,166	0,152	13,0
Std. 6	0	0,065	0,079	0,072	13,7
Positive C.		3,052	3,039	3,046	0,3
Internal C.		0,45	0,48	0,465	4,6

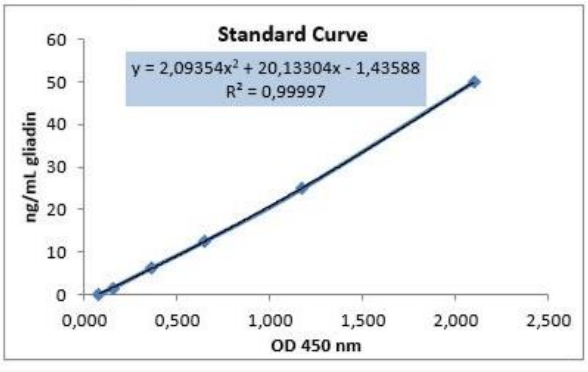

Figure 4. Example of the Standard Curve

13. Quality control

The performance of the assay can be controlled in two ways:

- **The Internal Control included in the kit must be used as a sample ready to use, without any further dilution. The analytical value of the internal control will be defined in the Certificate of Analysis of each specific batch. The value of an internal control batch obtained in an assay must have a maximum deviation of ±15 % of the analytical value of the same internal control batch defined in the Certificate of Analysis.**
- **The absorbance of the positive control must be above that obtained for Standard 1 (S1, 50 ng/mL of gliadin) and the absorbance of the S6 must be below that obtained for Standard 5 (S5, 1.56 ng/mL of gliadin).**

If any of these controls fail, 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) of the assay is 0.4 ppm gluten/0.2 ppm gliadin. The limit of quantification (LoQ) is 1.2 ppm gluten/0.6 ppm gliadin. The range of quantification of the assay is 1.2-200 ppm gluten (0.6-100 ppm gliadin). Depending on the sample dilution analyzed, the quantifiable amount 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)
1:20	1.2	20
1:50	1.6	50
1:100	3.1	100
1:200	6.2	200

NOTE: If a gluten value is obtained that is above the stated Upper Quantification Limit for the dilution used, further dilutions must be done and re-tested to obtain a valid result.

The limit of detection (LoD) of the swab sample method is 1.8 ng gliadin/cm^2 (3.6 ng gluten/ cm^2). However, this result cannot be extrapolated to a final concentration of gluten ppm on the surface.

Specificity

This test is based on the G12 and A1 monoclonal antibodies, which can specifically detect the presence of the immunotoxic fraction of the prolamins of **wheat** (gliadin), **rye** (secalin), **barley** (hordein) and some rare 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.

When the source of gluten is barley or rye, this test could show an occasional and slight overestimation (2.5%-37%) depending on the matrix and gluten concentration in the sample. Sporadically, the overestimation could be moderate (46%-85%).

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

- Shan L., et al., “Structural basis for gluten intolerance in celiac sprue”, *Science* 2002; 297:2275-9.
- 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.
- 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.

Appendix 1. Recommended Protocols

A. Extraction procedure for foods and drinks containing polyphenols, tannins or antioxidants

1.1. Homogenize, mill and/or triturate the sample.
1.2. Weigh 0.5 g or add 0.5 mL of sample in a test tube.
1.3. 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.
1.4. Add 5 mL of Extraction Solution.
1.5. Mix vigorously using a vortex mixer until complete disaggregation. With some samples, it can be helpful to pre-heat the sample for two minutes at 50 °C/122 °F and then vortex again until complete disaggregation.
1.6. Once completely disaggregated, use option 10.1.4 b) for incubation.
(40 minutes at 50 °C/122 °F) and follow the rest of the procedure as usual.

B. Extraction procedure for fatty foods.

1.1. Homogenize, mill and/or triturate the sample.
1.2. Weigh 0.5 g or add 0.5 mL of sample in a test tube.
1.3. Add 5 mL of Extraction Solution.
1.4. Use a weighing spoon or pallet to physically help the disaggregation of the sample until a suspension with particles below 2 mm is achieved. The nature of those samples will avoid the simple disaggregation by vortexing; therefore, an external help for that is needed. This procedure can render an increase of recovery up to 20%.
1.5. Once completely disaggregated, use option 10.1.4 a) for incubation [40 minutes at room temperature (15 to 25 °C /59 to 77 °F) with mild agitation] and follow the rest of the procedure as usual.