

**AOAC Official Method 2012.01  
Gliadin as a Measure of Gluten  
in Rice- and Corn-Based Foods**

**Enzyme Immunoassay Method  
Based on a Specific Monoclonal Antibody to the  
Potentially Celiac Toxic Amino Acid Prolamine Sequences  
First Action 2012  
Final Action 2016**

*Caution:* Cocktail solution necessary for sample preparation contains  $\beta$ -mercaptoethanol. Use a chemical hood for sample preparation. Stop solution contains 1 M sulfuric acid. Avoid skin and eye contact (*see* Material Safety Data Sheet).

*See* Table 2012.01 for the results of the interlaboratory study supporting acceptance of the method.

**A. Principle**

The method is based on an enzyme immunoassay format using a monoclonal antibody that can determine gliadin derived from wheat and related prolamins derived from rye and barley. The antibody binds to the potentially celiac toxic amino acid sequence QQPF (1) and to related sequences, which exist as motifs on all the gliadin subunits. The antibody detects prolamins in nonheated and heated food by using an additional specific extraction method (cocktail solution). No cross-reactivity exists to oats, maize, rice, millet, teff, buckwheat, quinoa, and amaranth.

Prolamins from food are extracted by using a cocktail solution containing  $\beta$ -mercaptoethanol and guanidine hydrochloride described by García et al. (2), following an extraction with 80% ethanol. After centrifugation, the supernatant is used in a second-step sandwich method. The analyte is incubated in monoclonal antibody-coated wells forming an antibody–antigen complex. In a second step, an antibody peroxidase (POD) conjugate reacts with the complex to form an antibody–analyte–antibody complex. A chromogen/substrate reaction with the immobilized POD labeled conjugate determines the bound analyte. Nonimmobilized components are removed by washing between steps. The response

of sample extracts is compared with response observed with calibrators.

**B. Apparatus**

Apparatus specified has been tested. Equivalent apparatus may be used.

(a) *Grindomix GM 200*.—For sample homogenization (Retsch GmbH, Haar, Germany).

(b) *Water bath*.—Gesellschaft für Labortechnik mbH (Burgwedel, Germany).

(c) *Bench-top centrifuge*.—Multifuge 3L-R, operating at 2500 rpm (Thermo Electron GmbH, Dreieich, Germany).

(d) *Glass tubes*.—10 mL; for extraction (Brand GmbH, Wertheim, Germany).

(e) *Polystyrol tubes*.—5 mL; for sample dilution (Sarstedt, Nümbrecht, Germany).

(f) *Microtiter plate reader*.—With 450 nm filter (Tecan Deutschland GmbH, Crailsheim, Germany).

(g) *Micropipet*.—Accurately delivering 100  $\mu$ L  $\pm$  1%.

(h) *Glassware*.—Wash bottle (1000 mL) and graduated cylinders.

(i) *Rotator 3100 CMV or equivalent*.—Fröbel Labortechnik (Lindau, Germany).

**C. Antibody Characteristics**

Antibodies must satisfy the following criteria:

(1) Bind to gliadin derived from wheat and to related prolamins derived from rye and barley

(2) Recognize the potential celiac toxic structure QQPF and related sequences

(3) Bind to the  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\Omega$ -gliadin motifs in nonheated and heated food, extracted by cocktail solution

(4) No binding to- oats, maize, rice, teff, buckwheat, quinoa, and amaranth

(5) Bind with high affinity to allow an LOD of 1.5 mg/kg gliadin or related prolamins

(6) Able to build a stable POD labeled conjugate, stable for more than 1 year

**Table 2012.01. Interlaboratory study results for gliadin by RIDASCREEN® Gliadin**

Matrix	Material		No. of labs (outliers)	Mean, mg/kg	Recovery, %	RSD <sub>r</sub> , %	RSD <sub>R</sub> , %
	Level, mg/kg						
Maize	168		19 (1)	141.8	84.4	20.8	28.6
Maize	35		20 (0)	36.8	105.0	37.7	40.3
Maize	79		18 (2)	74.1	93.8	14.2	32.4
Maize	0		20 (0)	8.3		32.0	41.5
Rice	41		18 (2)	34.7	84.6	18.3	25.6
Rice <sup>a</sup>	0			<1.5			
Rice	147		17 (1)	126.6	86.1	26.8	35.4
Wheat starch	14		20 (0)	12.5	89.3	26.8	40.7
Rice flour	13		20 (0)	14.1	108.5	37.4	38.1
Wheat starch	13.5		17 (0)	13.2	97.8	29.7	52.1
Maize flour <sup>a</sup>	<1.5			<1.5			
Maize flour <sup>a</sup>	<1.5			<1.5			

<sup>a</sup> Negative samples were not included in the statistical evaluation.

(7) Show reproducible affinity, sensitivity, specificity, and stability from batch to batch for more than 1 year

(8) Monoclonal antibodies are preferred; polyclonal antibodies can be used if they fulfill the same specificity criteria to react with wheat, rye, and barley to 100% and have no cross-reactivity to oat, maize, teff, and others

#### D. Reagents

Items (a)–(i) are available as a test kit (RIDASCREEN® Gliadin; R-Biopharm AG, Darmstadt, Germany). All reagents are stable for 18 months from date of manufacture at 2–8°C (36–46°F). Refer to kit label for current expiration. Equivalent antibodies may be used for (a) and (c) provided they satisfy characteristic criteria in C.

(a) *Antibody-coated microwell strips*.—Monoclonal antibodies are coated in 20 mM phosphate buffered saline (PBS), pH 6.0, onto a set of twelve 8-microwell strips (NUNC, Roskilde, Denmark), containing 0.01% sodium azide as preservative.

(b) *Wash buffer concentrate*.—100 mL/bottle, 10x concentrate. Contains a final concentration of 20 mM PBS (0.9% sodium chloride) with 0.1% Synperonic and 0.01% bronidox L as preservative.

(c) *Peroxidase-labeled antibody*.—One vial (1.2 mL, 11x concentrated).

(d) *Gliadin ready-to-use standards (antigen)*.—Six vials (1.3 mL each, ready to use). Prepared by Sigma gliadin or own preparation, dissolved in 60% ethanol at a concentration of 1 mg/mL. Solution is further diluted in 20 mM PBS–Tween (0.9% sodium chloride, 0.05% Tween 20) containing 0.22% fish gelatin (Sigma) to 0, 5, 10, 20, 40, and 80 ng/mL gliadin, calibrated to the Working Group on Prolamin Analysis and Toxicity (WGPAT) gliadin (86% highly purified gliadin from 40 different European wheat varieties).

(e) *Substrate*.—One vial, 7 mL (urea peroxide).

(f) *Chromogen*.—One vial, 7 mL (tetramethylbenzidine in methanol). Can be added either separately or mixed 1 + 1 with (e) before pipetting.

(g) *Stop solution*.—One vial, 14 mL (1 N H<sub>2</sub>SO<sub>4</sub>).

(h) *Sample dilution buffer*.—60 mL, 5x concentrate. Contains a final concentration of 20 mM PBS–Tween (0.9% sodium chloride, 0.05% Tween 20) with 0.22% fish gelatin (Sigma) and 0.01% Kathon as preservative.

(i) *Cocktail solution*.—One vial, 105 mL.

Recommended but not provided with the test kit:

(a) *Skim milk powder*.—Food quality.

(b) *Samples*.—Three control samples (powder), one nongliadin-containing sample (rice flour) and two prolamine-contaminated maize samples (A and B, concentration given by a certificate), which can be extracted with 60% ethanol and diluted further with the sample dilution buffer to control the test from run to run.

#### E. General Instructions

Store kit at 2–8°C (35–46°F). Let all kit components come to 20–25°C (68–77°F) before use.

Return any unused microwells to their original foil bag, reseal them together with the desiccant provided, and store at 2–8°C (35–46°F). The colorless chromogen is light-sensitive; therefore avoid exposure to direct light.

Include ready-to-use standards in duplicates to each run of diluted sample extracts in duplicates. Add the diluted antibody–POD conjugate (diluted by water) to all wells. Add substrate and chromogen simultaneously. Stop the reaction with stop solution,

and measure in a microtiter plate reader at 450 nm vs air within 30 min after stopping the reaction. Do not reuse wells of the plate.

Use separate pipet tips for each standard and each sample extract to avoid cross-contamination.

Use a multistep pipet for adding the conjugate, substrate/chromogen, and stop solution. Use a single tip for each of these components. Components and procedures of test kit have been standardized for use in this procedure. Do not interchange individual components between kits of different batches (lot numbers). Do not freeze any of the kit components.

Carefully dilute the components that are included in the kit as concentrates; avoid contaminations by airborne cereal dust or dirty laboratory equipment. Wear gloves during preparation and performance of the assay. Clean surfaces, glass vials, mincers, and other equipment with 60% ethanol. Carry out sample preparation in a room isolated from ELISA procedure. Check for prolamins contaminations of reagents and equipment.

#### F. Preparation of Test Samples

Weigh 5 g sample and grind to a powder as fine as possible to obtain maximal surface. Weigh 0.25 g of the solid ground sample or use 0.25 mL of a liquid sample in a 10 mL glass vial and add 2.5 mL cocktail. Close vial and mix well (avoid cross-contamination). If tannin- and polyphenol-containing samples (e.g., chocolate, chestnut, or buckwheat) are prepared, add an additional 0.25 g skim milk powder (food quality) to the sample–cocktail solution.

Incubate for 40 min at 50°C (122°F) in a water bath. Let sample cool down; then mix with 7.5 mL 80% ethanol. Close vial and shake for 1 h upside down or by a rotator at room temperature (20–25°C/68–77°F). Centrifuge 10 min at 2500 g at room temperature (20–25°C/68–77°F). Remove the supernatant (extract) in a screw-top vial and keep for testing.

Dilute the sample at least 1:12.5 (1 + 11.5, 0.1 + 1.15 mL) with the prepared sample dilution buffer (depending on the expected prolamins content of the sample). Dilute serially from the first dilution, if necessary mixing thoroughly each time before diluting further. Use 100 µL per well in the assay.

#### G. Preparation of Components Delivered with Kit

(a) *Sample diluent*.—Provided as a concentrate (5-fold). Only the amount which is actually needed should be diluted 1:5 (1 + 4) with distilled water (e.g., 3 mL concentrate + 12 mL distilled water, sufficient for the dilution of 10 samples). Make sure that the buffer is not contaminated with gliadin.

(b) *Antibody enzyme conjugate*.—(Bottle with red cap.) Provided as a concentrate (11-fold). Since the diluted enzyme conjugate solution has a limited stability, only the amount that is actually needed should be diluted. Before pipetting, the conjugate concentrate should be shaken carefully. For reconstitution, the conjugate concentrate is diluted 1:11 (1 + 10) with distilled water (e.g., 200 µL concentrate + 2.0 mL distilled water, sufficient for two microtiter strips). Take care that the water is not contaminated with gliadin.

(c) *Washing buffer*.—Provided as a 10-fold concentrate. Before use, the buffer must be diluted 1:10 (1 + 9) with distilled water (i.e., 100 mL buffer concentrate + 900 mL distilled water). Prior to dilution, dissolve any crystals formed by incubating the buffer in a water bath at 37°C (99°F). The diluted buffer is stable at 2–8°C (35–46°F) for 4 weeks.

## H. Determination

Bring all reagents to room temperature (20–25°C/68–77°F) before use. Do not allow microwells to dry between working steps. Insert a sufficient number of wells into the microwell holder for all standards and samples to be run. Record standard and sample positions.

Add 100 µL of each standard solution or prepared sample to separate wells, mix 10 s manually, and incubate for 30 min at room temperature (20–25°C/68–77°F). Dump the liquid out of the wells, and then tap the microwell holder upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. Fill all the wells with 250 µL diluted washing buffer and dump out the liquid again. Repeat two more times.

Add 100 µL of the finally diluted enzyme-labeled conjugate to each well, mix 10 s manually, and incubate for 30 min at room temperature (20–25°C/68–77°F). Dump the liquid out of the wells, and then tap the microwell holder upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. Fill all the wells with 250 µL diluted washing buffer and dump out the liquid again. Repeat two more times.

Add 50 µL substrate and 50 µL chromogen to each well. Mix gently by shaking the plate 10 s manually and incubate for 30 min at room temperature (20–25°C/68–77°F) in the dark. Positive wells should develop a blue color, indicating the presence of prolamins. Add 100 µL stop reagent to each well. Mix gently by shaking the plate manually. The color of positive prolamin-containing wells changes from blue to yellow.

## I. Reading

Read the results with a microtiter plate reader. Measure the absorbance at 450 nm. Read within 30 min against air after addition of stop solution.

## J. Calculations

Determine the gliadin content of each set of duplicate sample wells by reference to a calibration curve measured by the actual test run utilizing special computer software or semilogarithmic paper; plot absorbance of standards (linear scale) vs gliadin content of standards (logarithmic scale).

The standard calibration curve of the ELISA covers a range from 2.5 to 40 mg gliadin/kg sample, which corresponds to a range of 5–80 ng/mL gliadin in the calibrators. Convert the units ng gliadin/mL diluted sample to mg gliadin/kg sample as follows: Multiply the amount in ng/mL by the dilution factor. Divide the product by 1000 to achieve units of mg/kg. The dilution factor corresponds to the sample preparation and is usually 500; however, 1000 was used in this study. Absorbance below standard 2 (5 ng/mL gliadin) implies that the sample assayed is diluted too much or that no gliadin or gliadin below the LOQ is present in the sample.

Gluten content of a sample can be calculated from the gliadin value, as gliadin generally represents 50% of the proteins present in gluten. Gluten values can be expressed in mg/kg by multiplying the gliadin value by 2.

*Example calculation:* A sample was extracted with the recommended dilution factor of 500. The absorbance value of the sample corresponds to 10 ng/mL gliadin in the calibration curve. By multiplying the obtained value by the factor 500 leads to 5000 ng/mL, corresponding to 5 mg/kg gliadin, respectively, 0.0005% gliadin. To calculate the gluten content, multiply by factor 2, which results in 10 mg/kg gluten, respectively, 0.001% gluten. This sample is considered to be gluten-free because the gluten concentration is below 20 mg/kg gluten.

LOD was calculated by testing 10 blank samples/matrix; mean values and standard deviation (SD) were calculated. LOD was defined as mean + 3x SD.

LOQ was verified by analyzing 10 replicates of a food sample, which contains a gliadin content close to standard 2 [5 ng/mL × 500 (dilution factor) = 2.5 ppm gliadin]. In parallel standard 1 (= 0 ng/mL gliadin) was measured 10 times. The variation of standard 1 (absorbance value + 3x SD) was confirmed. The mean value – 3x SD was found significantly different from zero in consideration of the CV.

## K. Criteria for Acceptance of Standard Curve

The course of the calibration curve is shown in the Quality Assurance Certificate, enclosed in the test kit. In comparison with the certificate, higher values of the absorbance at 450 nm, especially for the zero calibrator, may be a result of insufficient washing or gliadin contamination. A further dilution and repeated measurement of the samples is recommended for absorbance values (450 nm) higher than standard 6. This additional dilution factor must be taken into consideration during calculation.

Indication of instability or deterioration of reagents is shown by any coloration of the chromogen solution prior to test implementation or if values of less than 0.6 absorbance units for standard 6 occur. SD of replicates should be less than 10%. Test controls offered by R-Biopharm should be measured in the reported ranges from run to run.

References: (1) Osman, A.A., Uhlig, H.H., Valdes, I., Amin, M., Mendez, E., & Mothes, T. (2001) *Eur. J. Gastroenterol. Hepatol.* **13**, 1189–1193

(2) García, E., Llorente, M., Hernando, A., Kieffer, R., Wieser, H., & Méndez, E. (2005) *Eur. J. Gastroenterol. Hepatol.* **17**, 529–539

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*Revised 2016 to update title as part of Final Action vote (change “foods containing wheat, rye, and barley” to “rice- and corn-based foods”); March 2017 to include modification of the wash solution to substitute thimerosal in the washing buffer by the mercury-free preserving agent bronidox L, D(b).*

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