CERTIFICATION

AOAC® *Performance Tested*SM

Certificate No.

061502

The AOAC Research Institute hereby certifies the test kit known as:

GlutenTox® Pro

manufactured by

Hygiena Diagnóstica España

P. I. Parque Plata, Calle Cañada Real 31-35

Camas, Sevilla 41900

Spain

This method has been evaluated in the AOAC® *Performance Tested Methods*SM Program and found to perform as stated by the manufacturer contingent to the comments contained in the manuscript. This certificate means that an AOAC® Certification Mark License Agreement has been executed which authorizes the manufacturer to display the AOAC *Performance Tested* SM certification mark along with the statement - "THIS METHOD'S PERFORMANCE WAS REVIEWED BY AOAC RESEARCH INSTITUTE AND WAS FOUND TO PERFORM TO THE MANUFACTURER'S SPECIFICATIONS" - on the above-mentioned method for a period of one calendar year from the date of this certificate (October 13, 2021 – December 31, 2022). Renewal may be granted at the end of one year under the rules stated in the licensing agreement.

Scott Coates

Scott Coates, Senior Director
Signature for AOAC Research Institute

October 13, 2021
Date

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KIT NAME(S)
GlutenTox® Pro

CATALOG NUMBERS
KIT 3000 (KT-5660; 25 analysis); KIT 3001 (KT-5288; 5 analysis)

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APPLICABILITY OF METHOD
Target analyte – Gluten

Matrixes – bread, rice flour, paté, rolled oat, yogurt, food-grade painted wood, plastic, rubber, sealed ceramic, stainless steel

Performance claims - The GlutenTox®Pro test kit is a quick and easy to use screening method for the detection of gluten in raw or cooked foods and on environmental surfaces.
The method is specific and reliable and provides sensitive and accurate test results comparable to AOAC OMA 2012.01.

REFERENCE METHOD
AOAC Official Methods of Analysis (OMA) 2012.01 "Gliadin as a Measure of Gluten in Foods Containing Wheat, Rye, and Barley" (11)

ORIGINAL CERTIFICATION DATE
June 26, 2015

CERTIFICATION RENEWAL RECORD
Renewed annually through December 2022

METHOD MODIFICATION RECORD
1. **December 2018 Level 2**
2. **November 2019 Level 1**

SUMMARY OF MODIFICATION
1. **Purchase and location change from Biomedal Avenida Américo Vespucio, 5-E, 1ª M-12, 41092 Sevilla, Spain to Hygiena Diagnóstica España P. I. Parque Plata, Calle Cañada Real 31-35, 41900 Camas, Sevilla, Spain.**
2. **Editorial/clerical changes.**

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NONE

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NONE

PRINCIPLE OF THE METHOD (1)
The GlutenTox®Pro method is an immunochromatographic assay for the detection of gluten in food and beverages (with non-hydrolyzed gluten) with different composition and levels of processing, from raw materials to processed food. In addition, the GlutenTox®Pro Test Kit can be used to control the cleanliness of food production zones through surface analysis, a prerequisite to prevent the risk of cross-contamination in the final product.

DISCUSSION OF THE VALIDATION STUDY (1)

The GlutenTox®Pro method did not show cross-reactivity to any of the compounds included in the list of *Validation Procedures for Quantitative Gluten ELISA Methods: AOAC Allergen Community Guidance and Best Practices*[4] used in the production of gluten-free products. The GlutenTox®Pro assay also did not show any interference, when tested with the compounds from the list in the presence of gluten. No unexpected results were obtained however gum-type samples can be difficult to analyze due to the thick paste formed when added to the extraction solution provided in the GlutenTox®Pro test kit. A warning to this type of samples has been included in the instructions for use.

The GlutenTox®Pro test kit performed as expected in the selected food matrixes (rice flour, bread, rolled oat, pâté and yogurt) and test conditions (spike level and detection threshold combinations), 5 ppm being the lowest concentration of gluten that can be detected with the kit.

In all matrixes tested, the GlutenTox®Pro method demonstrated 100 % specificity [probability of detection (POD) 0.00. confidence interval (CI) 0.00-0.11] at 0 ppm spiked level of gluten and 100 % sensitivity (POD 1.00., CI 0.89-1.00) at each spiked level of gluten and threshold level combinations. No false negative results were obtained in the food matrix study. The assay did not experience hook effect at any threshold level tested when the rice flour matrix was spiked at very high spiked levels of gluten (10,000 ppm).

In the incurred sample study, the incurred residue target level was approximately 25 ppm of gluten, the initial spiking level in the uncooked matrix was 50 ppm of gluten and a 78.2 % recovery was obtained when tested with the AOAC OMA 2012.01 method[11] (recovery could be between 50-150%).

The GlutenTox®Pro test kit performed as expected in the incurred bread sample and the results obtained in the incurred matrix study were consistent with those obtained in the selected food matrix study with bread. In both studies, false negative and/or overestimated results were not observed.

The results obtained when the GlutenTox®Pro test kit was tested with the selected environmental surfaces (food-grade painted wood, plastic, rubber, sealed ceramic and stainless steel) demonstrated a 100 % specificity (POD 0.00, CI0.00-0.11) at the unspiked level of gluten contamination and a 100 % sensitivity (POD 1.00., CI 0.89-1.00) at the high level of gluten contamination (400 ng/16 cm^2), in each of the environmental surfaces analyzed.

At the low level of gluten contamination (16 ng/16 cm^2), the GlutenTox®Pro assay was able to detect as little as 16 ng of gluten when analyzed with the environmental surface matrixes.

The lot-to-lot data, the accelerated stability data (10 days, 20 days, 35 days, 50 days and 90 days at 42ºC) and the real time stability data (3 months, 9 months, 18 months, 24 months, and 30 months at 22°C) showed evidence that the GlutenTox®Pro method is stable and can be consistently manufactured with reproducible quality.

Test kit variation data among 3 test kits of a single lot of GlutenTox®Pro test kit demonstrated no statistical difference in gluten detection between the test kits. Occasional slight overestimations are irrelevant in gluten analysis compared to a problem that could arise from false negatives or underestimations.

No false negative results were observed in the entire validation study.

Robustness data indicated that the GlutenTox®Pro assay remained unaffected by minor variations in procedural parameters with the exception of the amount of time that the test strip was left in the dilution sample solution before reading the result. Due to the test format, there must be sufficient time for the dilution sample solution to travel up the test strip, and this time cannot be shortened. The effect of decreasing the strip incubation time was not dependent of the amount of dilution sample solution used but this effect was smaller when coupled with an increased sample extraction time.

When the test strip was left in a smaller amount of dilution sample solution some invalid results appeared.

Table 3: GlutenTox®Pro Test Kit Incurred Matrix (Bread) – POD Results (1)

Matrix	Gluten Spiked Level*	Detection Threshold (ppm)	N[a]	Candidate			Ave. AOAC OMA 2012.01 results, ppm gluten, N=3	Variance (σ^2)
				x[b]	POD_C[c]	95% CI[d]		
Incurred Matrix (Bread)	0 ppm	5	30	0	0.00	0.00, 0.11	**<2.5**	–
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	39.1 ppm	5	30	30	1.00	0.89, 1.00	**39.1**	**1.2**
		10	30	30	1.00	0.89, 1.00		
		20	30	30	1.00	0.89, 1.00		
		40	30	0	0.00	0.00, 0.11		

*Gluten Spiked Level results after cooking the bread

[a]N = Number of test portions

[b]x = Number of positive test portions

[c]POD_C = Candidate method confirmed positive outcomes divided by the total number of trials

[d]95% Confidence Intervals

Table 4: GlutenTox®Pro Test Kit for Rice Flour – POD Results (1)

Matrix	Gluten Spiked Level	GlutenTox®Pro Detection Threshold (ppm gluten)	N[a]	Candidate			Ave. AOAC OMA 2012.01 results, ppm gluten, N=3	Variance (σ^2)
				x[b]	POD_C[c]	95% CI[d]		
Rice Flour	0 ppm	5	30	0	0.00	0.00, 0.11	**<2.5**	–
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	3 ppm	5	30	11	0.37	0.22, 0.54	**3.9**	**0.2**
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	8 ppm	5	30	30	1.00	0.89, 1.00	**8.8**	**0.2**
		10	30	3	0.10	0.03, 0.26		
		20	30	0	0.00	0.00, 0.11		

		40	30	0	0.00	0.00, 0.11		
	15 ppm	5	30	30	1.00	0.89, 1.00	**14.5**	**0.3**
		10	30	30	1.00	0.89, 1.00		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	25 ppm	5	30	30	1.00	0.89, 1.00	**21.5**	**1.8**
		10	30	30	1.00	0.89, 1.00		
		20	30	30	1.00	0.89, 1.00		
		40	30	0	0.00	0.00, 0.11		
	45 ppm	5	30	30	1.00	0.89, 1.00	**38.0**	**1.1**
		10	30	30	1.00	0.89, 1.00		
		20	30	30	1.00	0.89, 1.00		
		40	30	30	1.00	0.89, 1.00		
	10,000 ppm	5	10	10	1.00	0.72, 1.00	**8061.0**	**–**
		10	10	10	1.00	0.72, 1.00		
		20	10	10	1.00	0.72, 1.00		
		40	10	10	1.00	0.72, 1.00		

[a]N = Number of test portions
[b]x = Number of positive test portions
[c]POD_C = Candidate method confirmed positive outcomes divided by the total number of trials
[d]95% Confidence Intervals

Table 6: GlutenTox®Pro Test Kit for Bread – POD Results (1)

Matrix	Gluten Spiked Level	GlutenTox®Pro Detection Threshold (ppm gluten)	N[a]	Candidate			Ave. AOAC OMA 2012.01 results, ppm gluten, N=3	Variance (σ^2)
				x[b]	POD_c[c]	95% CI[d]		
Bread	0 ppm	5	30	0	0.00	0.00, 0.11	**<2.5**	**–**
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	3 ppm	5	30	0	0.00	0.00, 0.11	**2.3**	**0.1**
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	8 ppm	5	30	30	1.00	0.89, 1.00	**7.2**	**0.1**
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	15 ppm	5	30	30	1.00	0.89, 1.00	**14.0**	**1.5**
		10	30	30	1.00	0.89, 1.00		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	25 ppm	5	30	30	1.00	0.89, 1.00	**21.1**	**2.5**
		10	30	30	1.00	0.89, 1.00		
		20	30	30	1.00	0.89, 1.00		
		40	30	0	0.00	0.00, 0.11		
	45 ppm	5	30	30	1.00	0.89, 1.00	**38.5**	**2.4**
		10	30	30	1.00	0.89, 1.00		
		20	30	30	1.00	0.89, 1.00		
		40	30	30	1.00	0.89, 1.00		

[a]N = Number of test portions
[b]x = Number of positive test portions
[c]POD_C = Candidate method confirmed positive outcomes divided by the total number of trials
[d]95% Confidence Intervals

Table 7: GlutenTox®Pro Test Kit for Rolled Oat – POD Results (1)

Matrix	Gluten Spiked Level	GlutenTox®Pro Detection Threshold (ppm gluten)	N[a]	Candidate			Ave. AOAC OMA 2012.01 results, ppm gluten, N=3	Variance (σ^2)
				x[b]	POD_C[c]	95% CI[d]		
Rolled oat	0 ppm	5	30	0	0.00	0.00, 0.11	**<2.5**	–
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	3 ppm	5	30	0	0.00	0.00, 0.11	**2.7**	**0.0**
		10	30	2	0.07	0.02, 0.21		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	8 ppm	5	30	30	1.00	0.89, 1.00	**8.3**	**1.7**
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	15 ppm	5	30	30	1.00	0.89, 1.00	**12.6**	**1.0**
		10	30	30	1.00	0.89, 1.00		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	25 ppm	5	30	30	1.00	0.89, 1.00	**20.4**	**3.4**
		10	30	30	1.00	0.89, 1.00		
		20	30	30	1.00	0.89, 1.00		
		40	30	0	0.00	0.00, 0.11		
	45 ppm	5	30	30	1.00	0.89, 1.00	**41.0**	**3.5**
		10	30	30	1.00	0.89, 1.00		
		20	30	30	1.00	0.89, 1.00		
		40	30	30	1.00	0.89, 1.00		

[a]N = Number of test portions
[b]x = Number of positive test portions
[c]POD_C = Candidate method confirmed positive outcomes divided by the total number of trials
[d]95% Confidence Intervals

Table 8: GlutenTox®Pro Test Kit for Pâté – POD Results (1)

Matrix	Gluten Spiked Level	GlutenTox®Pro Detection Threshold (ppm gluten)	N[a]	Candidate			Ave. AOAC OMA 2012.01 results, ppm gluten, N=3	Variance (σ^2)
				x[b]	POD_C[c]	95% CI[d]		
Pâté	0 ppm	5	30	0	0.00	0.00, 0.11	**<2.5**	–
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	3 ppm	5	30	0	0.00	0.00, 0.11	**3.0**	**0.7**
		10	30	9	0.30	0.17, 0.48		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	8 ppm	5	30	30	1.00	0.89, 1.00	**9.2**	**0.4**
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	15 ppm	5	30	30	1.00	0.89, 1.00	**16.1**	**0.4**
		10	30	30	1.00	0.89, 1.00		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	25 ppm	5	30	30	1.00	0.89, 1.00	**27.6**	**36.8**
		10	30	30	1.00	0.89, 1.00		
		20	30	30	1.00	0.89, 1.00		
		40	30	0	0.00	0.00, 0.11		
	45 ppm	5	30	30	1.00	0.89, 1.00	**41.0**	**18.9**
		10	30	30	1.00	0.89, 1.00		
		20	30	30	1.00	0.89, 1.00		
		40	30	30	1.00	0.89, 1.00		

[a]N = Number of test portions
[b]x = Number of positive test portions
[c]POD_C = Candidate method confirmed positive outcomes divided by the total number of trials
[d]95% Confidence Intervals

Table 9: GlutenTox® Pro Test Kit for Yogurt – POD Results (1)

Matrix	Gluten Spiked Level	GlutenTox® Pro Detection Threshold (ppm gluten)	N[a]	Candidate			Ave. AOAC OMA 2012.01 results, ppm gluten, N=3	Variance (σ^2)
				x[b]	POD_C[c]	95% CI[d]		
Yogurt	0 ppm	5	30	0	0.00	0.00, 0.11	**<2.5**	–
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	3 ppm	5	30	0	0.00	0.00, 0.11	**3.2**	**0.0**
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	8 ppm	5	30	30	1.00	0.89, 1.00	**9.3**	**0.0**
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	15 ppm	5	30	30	1.00	0.89, 1.00	**16.6**	**2.4**
		10	30	30	1.00	0.89, 1.00		
		20	30	0	0.00	0.00, 0.11		
		40	30	0	0.00	0.00, 0.11		
	25 ppm	5	30	30	1.00	0.89, 1.00	**24.9**	**0.5**
		10	30	30	1.00	0.89, 1.00		
		20	30	30	1.00	0.89, 1.00		
		40	30	0	0.00	0.00, 0.11		
	45 ppm	5	30	30	1.00	0.89, 1.00	**38.2**	**1.5**
		10	30	30	1.00	0.89, 1.00		
		20	30	30	1.00	0.89, 1.00		
		40	30	30	1.00	0.89, 1.00		

[a]N = Number of test portions
[b]x = Number of positive test portions
[c]POD_C = Candidate method confirmed positive outcomes divided by the total number of trials
[d]95% Confidence Intervals

Table 10: GlutenTox® Pro Test Kit Environmental Surface– POD Results (1)

Matrix (16 cm^2)	Amount of Spiked Gluten (ng/16 cm^2)	N[a]	Candidate		
			x[b]	POD_C[c]	95% CI[d]
Food-grade painted wood	Blank 0	5	0	0.00	0.00, 0.43
	Low 16	30	25	0.83	0.66, 0.93
	High 400	5	5	1.00	0.57, 1.00
Plastic	Blank 0	5	0	0.00	0.00, 0.43
	Low 16	30	23	0.77	0.59, 0.88
	High 400	5	5	1.00	0.57, 1.00
Rubber	Blank 0	5	0	0.00	0.00, 0.43
	Low 16	30	26	0.87	0.70, 0.95
	High 400	5	5	1.00	0.57, 1.00
Sealed Ceramic	Blank 0	5	0	0.00	0.00, 0.43
	Low 16	30	25	0.83	0.66, 0.93
	High 400	5	5	1.00	0.57, 1.00
Stainless steel	Blank 0	5	0	0.00	0.00, 0.43
	Low 16	30	21	0.70	0.52, 0.83
	High 400	5	5	1.00	0.57, 1.00

[a]N = Number of test portions
[b]x = Number of positive test portions
[c]POD_C = Candidate method confirmed positive outcomes divided by the total number of trials
[d]95% Confidence Intervals

REFERENCES CITED

1. Síglez, M.A., Nocea, B., del Mar Pérez, M., Mª García, E., León, L., and Galera, C, Evaluation of the GlutenTox® Pro Test for the Detection of Gluten in Select Foods and Surfaces, AOAC® *Performance Tested*℠ certification number 061502.
2. AOAC Research Institute Validation Outline for GlutenTox® Pro Test for the Detection of Gluten, Approved – June 2015.
3. AOAC Guideline for Validation of Binary Chemistry Methods. Appendix I N, AOAC International, 2013.
4. Koerner, T.B., Abbott, M., Godefroy, S.B., Popping, B., Yeung, J.M., Diaz-Amigo, C., Roberts, J., Taylor, S.L., Baumert, J.L., Ulberth, F., Wehling, P., & Koehler, P., (2013) "*Validation Procedures for Quantitative Gluten ELISA Methods: AOAC Allergen Community Guidance and Best Practices".* J. AOAC Int. **96**(5):1033-1040. http://dx.doi.org/10.5740/jaoacint.13-043
5. ISO 3534-2:2006, Statistics- Vocabulary and symbols.
6. Codex Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten (1979) Codex Alimentarius, Codex Standard 118-1979, rev. 2008, pp. 1-3. http://www.codexalimentarius.org/download/standards/291/cxs_118e.pdf
7. Shan L, Molberg Ø, Parrot I, Hausch, F., Filiz, F., Gray, G.M., Sollid, L.M. Khosla, C. (2002) "Structural basis for gluten intolerance in celiac sprue" Science **297**(5590):2275–9. http://www.sciencemag.org/content/297/5590/2275.full
8. Comino, I., Real, A., Lorenzo, L de., Cornell, H., López-Casado, M.A., Barro, F., Lorite, P., Torres, M.A., Cebolla, A., & Sousa C. (2011) "Diversity in oat potential immunogenicity: basis for the selection of oat varieties with no toxicity in coeliac disease" Gut **60**(7):915-922. doi:10.1136/gut.2010.225268
9. Morón, B., Cebolla, A., Manyani, H., Alvarez-Maqueda, M., Megías, M., Thomas, M del C., López, M.C., & Sousa, C. (2008)"Sensitive detection of cereal fractions that are toxic to celiac disease patients by using monoclonal antibodies to a main immunogenic wheat peptide" Am. J. Clin. Nutr. **87**(2), 405-414. http://ajcn.nutrition.org/content/87/2/405.long
10. Morón, B., Bethune, M.T., Comino, I., Manyani, H., Ferragud, M., López, M.C., Cebolla, A., Khosla, C., & Sousa, C. (2008) "Toward the Assessment of Food Toxicity for Celiac Patients: Characterization of Monoclonal Antibodies to a Main Immunogenic Gluten Peptide" PLoS ONE **3**(5): e2294. doi:10.1371/journal.pone.0002294
11. AOAC Official Methods of Analysis (OMA) 2012.01 "Gliadin as a Measure of Gluten in Foods Containing Wheat, Rye, and Barley".
12. van Eckert, R., Berghofer, E., Ciclitira, P.J., Chirdo, F., Denery-Papini, S., Ellis, H.J., Ferranti, P., Goodwin, P., Immer, U., Mamone, G., Mendez, E., Mothes, T., Novalin, S., Osman, A., Rumbo, M., Stern, M., Thorell, L., Whim, A., & Wieser, H., (2006) "Towards a new gliadin reference material - isolation and characterization" J. Cereal Sci. 43(3):331-341.
13. Least Cost Formulations, Ltd., AOAC Binary Data Interlaboratory Study Workbook (2011) (http://lcfltd.com/aoac/aoac-binary-v2-2.xls)
14. Siglez, M.A., Cebolla, A., (2010) "Método de detección de gluten en superficies" Alimentaria **411**:67-70.
15. Porterfield and Capone (1984) "Application of Kinetic Models and Arrhenius Methods to Product Stability Evaluation" Medical Devices and Diagnostic Industry, pp 45-50.