CERTIFICATION

AOAC Research Institute

Performance Tested Methods[SM]

Certificate No.

052005

The AOAC Research Institute hereby certifies the method known as:

SENSISpec INgezim Gluten R5

manufactured by

Gold Standard Diagnostics Madrid S.A.
C/Hermanos Gracía Noblejas, 41 2ª planta
28037 MADRID, SPAIN

This method has been evaluated and certified according to the policies and procedures of the AOAC Performance Tested MethodsSM Program. This certificate indicates an AOAC Research Institute Certification Mark License Agreement has been executed which authorizes the manufacturer to display the AOAC Research Institute Performance Tested Methods[SM] certification mark on the above-mentioned method for the period below. Renewal may be granted by the Expiration Date under the rules stated in the licensing agreement.

Bradley A. Stawick, Senior Director
Signature for AOAC Research Institute

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Expiration Date	December 31, 2025

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RESPONSIBLE COMPANY
Gold Standard Diagnostics
C/Hermanos Gracía Noblejas, 41 2ª planta
28037 MADRID, SPAIN

METHOD NAME
SENSISpec INgezim Gluten R5

CATALOG NUMBER
30.GLU.K2

INDEPENDENT LABORATORY
Q Laboratories, Inc.
1930 Radcliff Drive
Cincinnati, Ohio USA

APPLICABILITY OF METHOD
Target Analyte(s) – Prolamin proteins from Gluten: gliadins from wheat, secalins from rye, and hordeins from barley

Matrixes – (0.25 g): gluten-free bead mix, oat flour
stainless steel (10 cm x 10 cm)

Performance claims - Detection and quantification of gliadin gluten-free bread and oats.
Qualitative detection of gliadin on stainless steel surfaces.

REFERENCE METHOD

AOAC OMA 2012.01 (2)

ORIGINAL CERTIFICATION DATE
May 22, 2020

CERTIFICATION RENEWAL RECORD
Renewed through December 2025.

METHOD MODIFICATION RECORD
1. **December 2023 Level 1**

SUMMARY OF MODIFICATION
1. **Rebranding company name change from Eurofins Ingenasa to Gold Standard Diagnostics Madrid.**

Under this AOAC *Performance Tested Methods*℠ License Number, 052005 this method is distributed by:
Eurofins Technologies, Eurofins Abraxis, Eurofins GeneScan, Eurofins GSD, Granotec, Nuscana

Under this AOAC *Performance Tested Methods*℠ License Number, 052005 this method is distributed as:
SENSISpec INgezim Gluten R5 (30.GLU.K2)

PRINCIPLE OF THE METHOD (1)
The method is based on a double antibody sandwich enzyme immunoassay using the R5 monoclonal antibody (mAb), which recognizes an epitope common to the prolamin fraction from wheat (gliadins), rye (secalins) and barley (hordeins). The assay involves the following steps:

1) The sample is ground (if required) and dissolved in solution buffer to bring relevant target molecules into solution.
2) An aliquot of 100 µL is then pipetted into the wells of the ELISA plate containing the bound -specific R5 antibodies. If gluten is contained in the sample, it will be bound by the antibodies on the plate.
3) Using washing buffer, the remains of the unbound material is washed out.
4) Add a volume of 100 µL of the R5 mAb conjugated with Horse-radish peroxidase (HRPO) to each well. This conjugated mAb will bind to the gluten previously captured by the coated antibody.
5) Using washing buffer, the unbound mAb conjugate is washed out.
6) 100 µL of TMB (Tetra-methylbenzidine) is added as a substrate of the HRPO enzyme (change of the colorless substrate solution into a blue product).
7) A stop Solution is added, to transform the blue color into yellow one. The intensity of yellow color can be measured with an ELISA reader (at 450 nm).
8) The gliadin content of the samples is determined by interpolation of their OD in the calibration curve. The calibration curve is established using the standard of the Prolamin Working Group (PWG). The obtained ng/mL gliadin value is converted to gluten content by using the formula explained in the calculations sheet.

DISCUSSION OF THE VALIDATION STUDY (1)
After the rigorous validation study to confirm the performance claims of the SENSISpec INgezim Gluten R5 Kit, all the results obtained were satisfactory according with the expected. More in detail, the linearity study analyzing five replicate test portions of the curve shows a very high reproducibility. The food matrix study demonstrates no differences, depending on the previous treatment of the sample, gliadin recovery after spiking the samples.
The recovery from the incurred bakery matrix samples tested was also as expected (98.4%). The cross-reactivity/interference study demonstrates that the assay does not produce false positive results with the selected matrixes and there are no interferences with them. As a final comment, we can indicate that the assay is valid for the detection of gluten in contaminated surfaces.
In addition, the data from the independent validation study support the product claims of the SENSISpec INgezim Gluten R5 method in fresh raw select foods and stainless steel surfaces.

Table 5. Results of the test of the samples from Matrix study by the candidate method and reference test. The table includes the information about: the mean of the mg of gliadin per kg of product obtained as a mean of the different replicates, the standard deviation, the coefficient of variation (CV or RDSr), the Bias and the % of recovery. (1)

Sample	Contamination mg/kg Gliadin	Method	Mean	Standard Deviation	RDSr	Bias	% Recovery
Gluten free bread mix	**0**	Candidate Method	<LOQ	NA	NA	NA	NA
		REFERENCE	*<LOQ*	*NA*	*NA*	*NA*	*NA*
	1.70	Candidate Method	1.60	0.41	25.63	-0.10	94.40
		REFERENCE	*<LOQ*	*NA*	*NA*	*NA*	*NA*
	2.00	Candidate Method	1.70	0.42	24.71	-0.33	83.30
		REFERENCE	*<LOQ*	*NA*	*NA*	*NA*	*NA*
	3.12	Candidate Method	2.80	0.56	20.00	-0.30	90.40
		REFERENCE	3.00	1.03	34.33	-0.12	*96.15*
	6.25	Candidate Method	5.78	0.95	16.40	-0.47	92.00
		REFERENCE	7.43	0.23	3.10	1.18	119
	12.50	Candidate Method	11.11	0.09	0.80	-1.39	89.00
		REFERENCE	12.19	0.21	1.80	-0.31	98.00
	20.00	Candidate Method	17.90	2.24	12.51	-2.10	89.50
		REFERENCE	24.20	0.70	2.89	4.20	121.00
Oat	**0**	Candidate Method	<LOQ	NA	NA	NA	NA
		REFERENCE	<LOQ	NA	NA	NA	NA
	1.70	Candidate Method	1.68	0.15	0.09	-0.02	99.12
		REFERENCE	<LOQ	NA	NA	NA	NA
	2.00	Candidate Method	2.13	0.16	0.07	0.13	106.66
		REFERENCE	<LOQ	NA	NA	NA	NA
	3.12	Candidate Method	3.62	0.46	0.13	0.50	116.15
		REFERENCE	3.60	0.52	14.44	0.48	115.38
	6.25	Candidate Method	6.88	0.17	2.50	0.63	110.00
		REFERENCE	7.65	0.05	0.60	1.40	122.00
	12.50	Candidate Method	13.01	0.15	1.20	0.51	104.00
		REFERENCE	16.66	1.80	10.80	4.16	133.00
	20.00	Candidate Method	20.60	1.68	0.08	0.60	103.00
		REFERENCE	23.00	0.50	2.10	3.00	115.00
White rice flour	**100.00**	Candidate Method	98.20	3.06	3.10	-1.78	98.20
White rice flour	**100.00**	Candidate Method	108.03	9.20	8.50	8.00	108.03
White rice flour	**100.00**	Candidate Method	103.00	2.05	2.00	3.84	103.00
White rice flour	**100.00**	Candidate Method	98.60	1.90	1.90	-1.40	98.60
White rice flour	**100.00**	Candidate Method	95.70	2.80	2.90	-4.30	95.70

Table 7. Summary of the results obtained with the incurred gluten-free bread samples. The table shows extractions from 1 to 10 that are analyzed by the candidate method (SENSISpec INgezim Gluten R5) and extractions from 11 to 13 that are analyzed by the reference method. In the table is included the Optical density (OD at 450nm) obtained, the ppm of gliadin, the mean of OD of the different extractions, the standard deviation and de % of recovery. *NA: Not applicable (1)

Test	Contamination level	Extraction	OD 450 nm	ppm gliadin	Mean	Std dev σ	% Recovery
SENSISpec INgezim Gluten R5	0ppm	Extr 1	0.113	<LOQ	NA	NA	NA
		Extr 2	0.131	<LOQ			
		Extr 3	0.139	<LOQ			
		Extr 4	0.107	<LOQ			
		Extr 5	0.125	<LOQ			
		Extr 6	0.097	<LOQ			
		Extr 7	0.102	<LOQ			
		Extr 8	0.099	<LOQ			
		Extr 9	0.109	<LOQ			
		Extr 10	0.16	<LOQ			
Reference Method OMA 2012.01		Extr 11	0.143	<LOQ	NA	NA	NA
		Extr 12	0.375	<LOQ			
		Extr 13	0.139	<LOQ			
SENSISpec INgezim Gluten R5	5 ppm	Extr 1	1.219	5.55	4.92	0.41	98.40
		Extr 2	1.181	5.28			
		Extr 3	1.098	4.73			
		Extr 4	1.142	5.02			
		Extr 5	1.158	5.13			
		Extr 6	1.029	4.29			
		Extr 7	1.081	4.62			
		Extr 8	1.078	4.60			
		Extr 9	1.198	5.40			
		Extr 10	1.081	4.62			
Reference Method OMA 2012.01		Extr 11	0.868	5.89	6.14	0.38	122.80
		Extr 12	0.944	6.59			
		Extr 13	0.876	5.96			

Table 10. The table shows the results of the test of the samples from the surfaces. (1)

Matrix	Allergen	N[a]	Contamination level	1:12.5 Dilution		
				x[b]	POD_C[c]	95% CI
Stainless Steel Environmental Surfaces	Gluten	5	0 µg/mL/100cm^2	0	0.00	0.00, 0.43
		30	0.4 µg/mL/100cm^2	15	0.5	0.33, 0.66
		5	2 µg/mL/100cm^2	5	1.00	0.57, 1.00

[a]N = Number of test portions
[b]x = Number of positive test portions
[c]POD_C = 1:12.5 Dilution positive outcomes divided by the total number of trials

REFERENCES CITED

1. Romero, C., Venteo, A., González, I., Hevia, E., Rebollo, B., SENSISpec INgezim Gluten R5 (30.GLU.K2), AOAC *Performance Tested Methods*^{SM} certification number 052005.
2. AOAC OMA 2012.01.