



CERTIFICATION

AOAC Research Institute *Performance Tested Methods*SM

Certificate No.

042301

The AOAC Research Institute hereby certifies the method known as:

GlutenTox ELISA Rapid G12

manufactured by

Hygiena Diagnóstica España

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A handwritten signature in black ink, appearing to read "Bradley A. Stawick".

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

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METHOD NAME GlutenTox ELISA Rapid G12	CATALOG NUMBER KIT3075
INDEPENDENT LABORATORY Q Laboratories 1930 Radcliff Drive Cincinnati, OH 45204	APPLICABILITY OF METHOD Target analyte – Gluten from wheat, barley, and rye flour Matrixes – (0.5 g) – soy flour, corn bread, seasoning mix, rolled oats, evaporated milk, and gluten free baked bread Performance claims – The GlutenTox ELISA Rapid G12 test kit is designed to detect and quantify gluten in processed and non-processed foods listed above at a range of 1.2 - 200 mg/kg gluten. This range of quantitation is suitable for proposed gluten-free monitoring in the United States and is compliant with current EU regulations and Codex Alimentarius definitions.
ORIGINAL CERTIFICATION DATE April 12, 2023	CERTIFICATION RENEWAL RECORD Renewed through December 2025.
METHOD MODIFICATION RECORD NONE	SUMMARY OF MODIFICATION NONE
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PRINCIPLE OF THE METHOD (1)

The GlutenTox ELISA Rapid G12 method is a sandwich ELISA assay that can be used to detect and quantify gluten in food samples.

To solubilize the gluten present in the sample's matrix, the extraction solution (UGES) provided in the kit is added to the food sample.

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 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.

DISCUSSION OF THE VALIDATION STUDY (1)

The GlutenTox ELISA Rapid G12 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*” (1) or those added to the AOAC Research Institute Performance Tested MethodsSM program: *PTM validation of the GlutenTox ELISA Rapid G12 Kit in Select Foods*, version 10, 27 Sep 2021 used in the production of gluten-free products. Four compounds that had tested positive in the prescreening evaluation with the AOAC OMA 2012.01 method (6) (oat flour, romano bean flour, fava bean flour and Lima bean flour) also tested >LOQ with the GlutenTox ELISA Rapid G12 test kit and were not retested. For the sake of finding out if the positivity of these matrices was due to a gluten contamination during the manufacturing process or to a cross-reaction, further analysis was carried out using the same matrices in bean format (and rolled oats) and grinding them in the lab before performing the tests to minimize the risk of a gluten contamination. Definitely, the GlutenTox ELISA Rapid test kit did not show cross reactivity with these matrices. Therefore, it can be confirmed that the previous positive results were due to a gluten contamination. The GlutenTox ELISA Rapid G12 assay also did not show any interference, when tested with the required compounds for testing in the presence of gluten. No unexpected results were obtained (the lima bean matrix included in the interference study was that in bean format and subsequently ground).

The GlutenTox ELISA Rapid G12 test kit performed as expected when 6 additional wheat flour varieties were tested in rice flour and positive results were obtained in all wheat cultivars analyzed. However, with the Einkorn Wheat Flour (*Triticum monococcum*) variety, a recovery result lower than expected was obtained. Further studies would be needed to determine if this is due to a lower gluten:protein ratio.

The GlutenTox ELISA Rapid G12 test kit performed as expected in the selected food matrixes (gluten free soy flour, corn bread, seasoning mix, rolled oats and evaporated milk), spike levels of gluten with wheat flour, and in both Hygiene (method developer) and the independent laboratory (only the corn bread and seasoning mix matrixes were tested), obtaining comparable results.

In all matrixes tested at different spike levels with barley and rye flours, the GlutenTox ELISA Rapid G12 assay performed as expected (meeting performance claims for recovery and repeatability, mainly with barley flour) or showing slight (<28%) to moderate (46% or 85%) overestimation depending on the matrix, source of gluten contaminant and gluten concentration.

Results obtained in the method developer incurred matrix study with wheat, barley and rye flours indicate that the assay performed as expected (meeting performance claims for recovery and repeatability, mainly with wheat and rye flours) or showing slight (25% or 37%) to moderate (49%) overestimation depending on the source of gluten contaminant and gluten concentration. These data are comparable to those obtained in the incurred sample study of the independent laboratory where the GlutenTox ELISA Rapid G12 method performed as expected with wheat and rye flours and showed a slight (11%) to moderate (77%) overestimation with barley at 20 mg/kg and 30 mg/kg spike levels of gluten, respectively.

Nevertheless, this occasional overestimation of gluten from barley or rye is less important factor in gluten analysis for the people suffering from celiac disease, since possible problems from false negatives or underestimations could be much worse.

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

The GlutenTox ELISA Rapid G12 assay performed as expected in the calibration study in all dilutions. To minimize the trend of a non-random pattern found in the higher analyte concentrations of each dilution, a suitable dilution should be made according to the expected amount of gluten in the sample.

The intermediate precision study demonstrated that the design 2b and the contribution of the Analyst/Day/Calibration as a single confounded factor to the variance were appropriate when the GlutenTox ELISA Rapid G12 assay was tested with the incurred bread matrix.

In this study the overall RSD for the method was in accordance with the acceptance criteria, even was mathematically reduced by over 4% when the variance of the ELISA component was divided by the number of replicates tested (two ELISA wells per test portion).

The overall LOQ_{est} validated of the GlutenTox ELISA Rapid G12 test kit by the method developer in the selected matrixes performed as expected, showing an excellent correlation with the overall LOD-LOQ_{est} (according to the standard deviation of blank samples). These results are in line and are consistent with the LOD and LOQ values obtained from the independent laboratory (calculated from the linear regression model) using three matrixes and four spike concentration levels of gluten from wheat flour (LOD = 0.4 mg/kg gluten and LOQ = 1.2 mg/kg gluten).

Table 2: GlutenTox ELISA Rapid G12-Food matrix study with Wheat Flour from candidate and from independent laboratory (analyst 1 and analyst 2) (1)

Matrix	Target Contamination Level (mg/kg)	Candidate				
		Mean Concentration Obtained (mg/kg)	Recovery	Bias	S _r	RSD _r
Soy flour	0	-0.05	< LOD	-0.05	0.187	-410.941
	5	4.51	90.25	-0.49	0.648	14.361
	10	7.63	76.32	-2.37	1.031	13.519
	20	19.46	97.30	-0.54	1.719	8.833
Corn Bread	0	-0.14	< LOD	-0.14	0.271	-190.988
	5	6.20	123.95	1.20	0.730	11.793
	10	9.77	97.70	-0.23	0.684	7.001
	20	20.18	100.91	0.18	0.687	3.408
Seasoning Mix	0	0.07	< LOD	0.07	0.190	280.276
	5	4.37	87.39	-0.63	0.222	5.099
	10	9.51	95.08	-0.49	0.725	7.635
Rolled Oats	0	-0.05	< LOD	-0.05	0.170	-370.012
	5	5.70	113.96	0.70	0.359	6.307
	10	9.13	91.31	-0.87	1.126	12.333
	20	20.85	104.26	0.85	1.005	4.822
Evaporated Milk	0	0.28	< LOD	0.28	0.029	10.294
	5	3.13	62.54	-1.87	0.035	1.107
	10	6.58	65.82	-3.42	0.107	1.624
	20	14.31	71.56	-5.69	0.543	3.796
Independent laboratory, analyst 1						
Matrix	Target Contamination Level (mg/kg)	Mean Concentration Obtained (mg/kg)	Recovery	Bias	S _r	RSD _r
Corn Bread	0	0.328	/	0.328	0.015	4.482
	5	4.755	95.10	-0.245	0.520	10.938
	10	7.643	76.43	-2.357	0.570	7.452
	20	20.877	104.39	0.877	2.473	11.844
Seasoning Mix	0	0.197	/	0.197	0.029	14.638
	5	3.979	79.589	-1.021	0.182	4.582
	10	11.578	115.780	1.578	0.369	3.189
	20	21.268	106.340	1.268	1.320	6.206
Independent laboratory, analyst 2						
Matrix	Target Contamination Level (mg/kg)	Mean Concentration Obtained (mg/kg)	Recovery	Bias	S _r	RSD _r
Corn Bread	0	0.293	/	0.293	0.029	9.814
	5	4.683	93.667	-0.317	0.182	3.894
	10	7.561	75.617	-2.438	0.566	7.486
	20	20.916	104.580	0.916	2.431	11.623
Seasoning Mix	0	0.163	/	0.163	0.035	21.443
	5	4.165	78.833	-1.073	0.164	4.165
	10	11.452	114.517	1.452	0.314	2.739
	20	20.984	104.922	0.984	1.559	7.430

Table 3: GlutenTox ELISA Rapid G12-Food matrix study with Barley and Rye Flours from candidate (1)

Matrix/Contaminant	Target Contamination Level (mg/kg)	Candidate				
		Mean Concentration Obtained (mg/kg)	Recovery	Bias	S _r	RSD _r
Soy flour Barley flour	0	0.098	< LOD	0.098	0.059	60.345
	10	15.374	153.736	5.374	0.978	6.359
	20	31.779	158.897	11.779	1.276	4.014
Corn Bread Barley flour	0	0.098	< LOD	0.098	0.041	41.214
	10	13.083	130.832	3.083	2.051	15.675
	20	34.097	170.484	14.097	4.684	13.738
Seasoning Mix Barley flour	0	0.093	< LOD	0.093	0.098	105.809
	10	8.067	80.674	-1.933	0.123	1.529
	20	17.307	86.534	-2.693	0.896	5.176
Rolled Oats Barley flour	0	0.114	< LOD	0.114	0.068	59.686
	10	13.076	130.760	3.076	1.198	9.162
	20	28.776	143.880	8.776	2.656	9.231
Evaporated Milk Barley flour	0	0.039	< LOD	0.039	0.023	59.237
	10	18.737	187.370	8.737	1.408	7.516
	20	43.888	219.441	23.888	3.981	9.071

Matrix/Contaminant	Target Contamination Level (mg/kg)	Candidate				
		Mean Concentration Obtained (mg/kg)	Recovery	Bias	S _r	RSD _r
Soy flour Rye flour	0	0.138	< LOD	0.138	0.040	28.893
	10	18.206	182.064	8.206	3.064	16.829
	20	35.376	176.878	15.376	1.501	4.244
Corn Bread Rye flour	0	0.234	< LOD	0.234	0.074	31.512
	10	19.220	192.199	9.220	1.378	7.171
	20	55.572	277.861	35.572	7.895	14.206
Seasoning Mix Rye flour	0	0.086	< LOD	0.086	0.054	62.831
	10	18.524	185.243	8.524	3.054	16.484
	20	33.961	169.806	13.961	6.723	19.797
Rolled Oats Rye flour	0	0.125	< LOD	0.125	0.107	86.002
	10	13.890	138.901	3.890	1.803	12.977
	20	32.382	161.912	12.382	2.613	8.069
Evaporated Milk Rye flour	0	0.125	< LOD	0.125	0.095	76.082
	10	18.129	181.292	8.129	0.183	1.008
	20	36.231	181.157	16.231	0.952	2.628

Table 4: GlutenTox ELISA Rapid G12 – Incurred matrix study from candidate and from independent laboratory (analyst 1 and analyst 2) (1)

Matrix/Contaminant	Target Contamination Level (mg/kg)	Candidate				
		Mean Concentration Obtained (mg/kg)	Recovery	Bias	S _r	RSD _r
Baked Bread Wheat flour	0	0.084	< LOD	0.084	0.033	39.11
	20	13.976	69.882	-6.024	1.125	8.047
	30	21.276	70.921	-8.724	1.909	8.974
Baked Bread Barley flour	0	0.080	< LOD	0.080	0.077	95.75
	20	41.143	205.717	21.143	7.191	17.478
	30	67.282	224.273	37.282	8.953	13.307
Baked Bread Rye flour	0	0.078	< LOD	0.078	0.041	52.89
	20	37.540	187.699	17.540	6.943	18.494
	30	43.646	145.486	13.646	7.553	17.305
Independent laboratory, analyst 1						
Matrix/Contaminant	Target Contamination Level (mg/kg)	Mean Concentration Obtained (mg/kg)	Recovery	Bias	S _r	RSD _r
Baked Bread Wheat flour	0	0.141	/	0.141	0.035	24.992
	20	10.576	52.881	-9.424	1.077	10.179
	30	16.650	55.501	-13.350	1.639	9.846
Baked Bread Barley flour	0	0.141	/	0.141	0.035	24.992
	20	33.151	165.755	13.151	5.121	15.446
	30	79.338	264.460	49.338	17.988	26.673
Baked Bread Rye flour	0	0.141	/	0.141	0.035	24.992
	20	27.954	139.768	7.954	5.651	20.214
	30	40.598	135.325	10.578	5.024	12.374
Independent laboratory, analyst 2						
Matrix/Contaminant	Target Contamination Level (mg/kg)	Mean Concentration Obtained (mg/kg)	Recovery	Bias	S _r	RSD _r
Baked Bread Wheat flour	0	0.096	/	0.096	0.032	32.787
	20	10.654	53.271	-9.346	1.082	10.156
	30	16.749	55.828	-13.251	1.596	9.528
Baked Bread Barley flour	0	0.096	/	0.096	0.032	32.787
	20	33.385	166.927	13.385	5.189	15.543
	30	80.039	266.796	50.039	18.356	22.934
Baked Bread Rye flour	0	0.096	/	0.096	0.032	32.787
	20	27.746	138.728	7.746	6.021	21.700
	30	41.013	136.709	11.013	5.109	12.457

Table 5 GlutenTox ELISA Rapid G12.- LOD-LOQ_{est} study (1)

Blank matrixes: Concentration (mg/kg gluten)						
Replicate	Soy flour	Corn bread	Rolled oats	Seasoning mix	Evaporated milk	
1	0.256	0.237	0.373	0.419	0.468	
2	0.444	0.419	0.298	0.174	0.241	
3	0.453	0.481	0.390	0.381	0.392	
4	0.319	0.281	0.022	0.423	0.121	
5	0.436	0.499	0.369	0.219	0.361	
6	0.104	0.231	0.361	0.436	0.348	
7	0.440	0.445	0.161	0.407	0.263	
8	0.478	0.423	0.407	0.306	0.348	
9	0.256	0.192	0.419	0.361	0.122	
10	0.436	0.423	0.269	0.011	0.383	
Mean	0.362	0.363	0.307	0.314	0.305	
SDr	0.123	0.115	0.127	0.139	0.116	
LOD: Mean + 3.3 SDr	0.768	0.742	0.725	0.772	0.686	
Overall LOD = 0.738 mg/kg						
LOQ _{est} : Mean + 10 SDr	1.592	1.512	1.575	1.702	1.460	
Overall LOQ _{est} = 1.568 mg/kg						

Table 6 GlutenTox ELISA Rapid G12.- LOQ_{est} validation (1)

Replicate	Spiked matrixes at LOQ _{est} : Concentration (mg/kg gluten)				
	Soy flour	Corn bread	Rolled oats	Seasoning mix	Evaporated milk
1	1.434	1.475	2.207	1.396	1.011
2	1.456	1.541	1.541	1.545	1.102
3	1.493	2.009	2.001	1.707	1.140
4	1.697	1.668	2.089	1.249	1.179
5	1.438	1.781	1.893	1.348	1.179
6	1.181	2.014	2.226	1.951	1.058
7	1.420	1.602	1.908	1.747	1.089
8	1.574	1.517	1.810	1.712	1.110
9	1.285	1.640	1.614	1.519	1.071
10	1.848	1.682	1.898	1.541	1.007
Mean	1.483	1.693	1.919	1.571	1.095
SDr	0.191	0.190	0.226	0.211	0.061
RSD, %	12.857	11.214	11.804	13.450	5.554
Recovery%	93	112	122	92	75

Overall LOQ_{est} = 1.552 mg/kg

Table 7. GlutenTox ELISA Rapid G12.- LOD-LOQ_{est} study (1)

Replicate	Blank baked bread matrixes (incurred): Concentration (mg/kg gluten)		
	Wheat flour study	Barley flour study	Rye flour study
1	0.063	0.189	0.091
2	0.073	0.006	0.069
3	0.146	0.051	0.116
4	0.022	0.144	0.003
5	0.105	0.055	0.112
6	0.073	0.153	0.005
7	0.105	0.013	0.096
8	0.078	0.010	0.088
9	0.104	0.176	0.107
10	0.073	0.004	0.096
Mean	0.084	0.080	0.078
SDr	0.033	0.077	0.041
LOD: Mean + 3.3 SDr	0.193	0.333	0.215
Overall LOD = 0.247 mg/kg			
LOQ _{est} : Mean + 10 SDr	0.414	0.845	0.493
Overall LOQ _{est} = 0.584 mg/kg			

Table 8. GlutenTox ELISA Rapid G12 – Selectivity study. (1)

Compounds	GlutenTox ELISA Rapid G12		
	Un-spiked	20 ppm	
	Result (mg/kg gluten)	Result (mg/kg gluten)	Correctness
Almond Flour	Below LOQ	17.69	-12%
Amaranth flour	Below LOQ	18.27	-11%
Arrowroot	Below LOQ	18.86	-6%
Black bean flour	Below LOQ	19.89	-1%
Brown rice flour	Below LOQ	19,82	-1%
Buckwheat flour	Below LOQ	21.06	5%
Chestnut flour	Below LOQ	21.09	5%
Coconut flour	Below LOQ	23.24	16%
Ground Coffee	Below LOQ	19.76	-1%
Corn starch/meal	Below LOQ	20.54	3%
Dried fruits	Below LOQ	18.91	-5%
Egg powder	Below LOQ	20.37	2%
Fava bean flour	9.82	24.06	19%
Fava beans, ground*	Below LOQ	18.35	-8%
Flax seed flour	Below LOQ	22.09	10%
Green pea flour	Below LOQ	19.69	-2%
Guar gum (1:10)	Below LOQ	21.85	8%
Hazelnut flour	Below LOQ	19.18	-5%
Lentil flour	Below LOQ	19.09	-5%
Lima bean flour	297	-	-
Lima beans, ground*	Below LOQ	16.82	-16%
Milk powder	Below LOQ	20.44	1%
Milk (whole, liquid)	Below LOQ	14.82	-26%
Millet flour	Below LOQ	20.35	3%
Oat flour	2.79	17.38	-13%
Oats, rolled*	Below LOQ	20.85	4%
Parsley flakes	Below LOQ	22.14	10%
Pork sausage	Below LOQ	19.76	-3%
Potato starch	Below LOQ	23.71	19%
Quinoa flour	Below LOQ	19.18	-4%
Romano bean flour	6.06	23.68	18%
Romano beans, ground*	Below LOQ	18.53	-7%
Sorghum flour	Below LOQ	19.72	-1%
Soya flour	Below LOQ	22.76	13%
Sweet rice flour	Below LOQ	19.85	-1%
Tapioca flour	Below LOQ	19.11	-4%
Ground Tea	Below LOQ	15.39	-23%
White bean flour	Below LOQ	15.68	-22%
White rice flour	Below LOQ	18.64	-7%
Xanthan gum (1:20)	Below LOQ	18.47	-9%
Yellow pea flour	Below LOQ	24.17	19%

*Indicates commodities ground into meal from bean/oat material and re-tested.

Table 9. GlutenTox ELISA Rapid G12 – Selectivity study. Rice flour spiked at 20 mg/kg gluten from other sources of gluten (1)

Compounds	GlutenTox ELISA Rapid G12		
	Un-spiked	20 ppm	
	Result (mg/kg gluten)	Result (mg/kg gluten)	Correctness
Einkorn Wheat Flour (<i>Triticum monococcum</i>)	-	7.22	-64%
Khorasan Wheat flour (<i>Triticum turgidum</i>)	-	18.29	-9%
Spelt Wheat Flour (<i>Triticum spelta</i>)	-	23.84	18%
Triticale Flour (xTriticosecale)	-	8.39	-59%
Durum Wheat Flour (<i>Triticum durum</i>)	-	23.81	19%
Emmer Wheat Flour (<i>Triticum dicoccon</i>)	-	22.72	4%

REFERENCES CITED

1. Salagre, C., Lopez, A., and Galera, C., Validation of the GlutenTox® ELISA Rapid G12 for Determination of Gluten in Select Non-Heat Processed Matrixes and Heat Processed Matrixes, AOAC *Performance Tested*SM certification number 042301. Approved April 12, 2023.