GlutenTox® ELISA Competitive G12

KIT3012

Index

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1. Scope and Description of the Product

GlutenTox® ELISA Competitive G12 is an immunosorbent assay for the determination of the immunotoxic fraction of gluten that is harmful to celiac patients and can cause issues for gluten-sensitive people. This test is suitable for quantifying gluten in hydrolyzed foods. The present report describes the validation process and its results.

GlutenTox® ELISA Competitive G12 is based on the G12 antibody, which specifically recognizes the 33-mer peptide which is the most immunotoxic fraction of gluten [1]. The 33-mer is a peptide within the α-gliadin molecule that triggers most of the immune response in celiac patients [2].

2. Analytical Validation

The acceptance criteria defined for this analytical validation has been defined to meet the acceptance criteria for quantitative methods required by the AOAC in their document entitled *Guidelines for Standard Methods Performance Requirements*. We have applied even more restrictive values to assure the compliance.

A) Intra-Assay Variation

The intra-assay variation was determined by testing one standard curve in 12 replicates. The standard curve is composed of 5 standards (1000, 500, 250, 125 and 25 ng/ml hydrolyzed gliadin). Tables 1 (absorbances) and 2 (quantifications) and Figure 1 summarize the results of the intra-assay experiments.

Table 1: Analytical intra-assay variation of GlutenTox® ELISA Competitive G12. Absorbance values at 450 nm.

Hydrolyzed gliadin (ng/ml)	Stdr.	1	2	3	4	5	6	7	8	9	10	11	12	Average	SD	%CV
1000	S1	0.85	0.93	0.91	1.02	0.93	0.96	0.93	0.98	1.04	1.07	1.11	1.13	0.99	0.09	9%
500	S2	1.32	1.17	1.23	1.30	1.34	1.23	1.31	1.33	1.38	1.30	1.49	1.53	1.33	0.10	8%
250	S3	1.58	1.44	1.50	1.50	1.50	1.59	1.57	1.58	1.61	1.63	1.72	1.77	1.58	0.09	6%
125	S4	1.82	1.68	1.77	1.76	1.80	1.88	1.90	1.83	1.88	1.88	2.05	2.12	1.86	0.12	7%
25	S5	2.13	2.12	2.29	2.23	2.18	2.28	2.29	2.34	2.27	2.33	2.49	2.50	2.29	0.12	5%
2000	C +	0.55	0.56	0.58	0.60	0.63	0.63	0.65	0.61	0.70	0.69	0.71	0.66	0.63	0.05	8%
0	C -	2.70	2.59	2.63	2.55	2.58	2.50	2.61	2.65	2.45	2.70	2.86	2.83	2.64	0.12	5%
															Average	**7%**

Table 2: Analytical intra-assay variation of GlutenTox® ELISA Competitive G12. Quantifications (ng/ml hydrolyzed gliadin).

Hydrolyzed gliadin (ng/ml)	Stdr.	1	2	3	4	5	6	7	8	9	10	11	12	Aver-age	SD	%CV	% Recovery
1000	S1	978	974	1005	1011	1009	914	974	1012	988	937	990	971	980.26	30.11	3%	98%
500	S2	530	524	491	476	447	583	506	484	500	562	485	503	507.60	37.56	7%	102%
250	S3	259	252	255	265	295	255	280	253	269	245	281	288	266.53	16.01	6%	107%
125	S4	111	120	124	122	116	108	108	126	115	118	113	104	115.42	7.00	6%	92%
25	S5	26	25	25	25	25	27	26	25	26	26	26	27	25.69	0.62	2%	103%
															Average	**5%**	**100%**

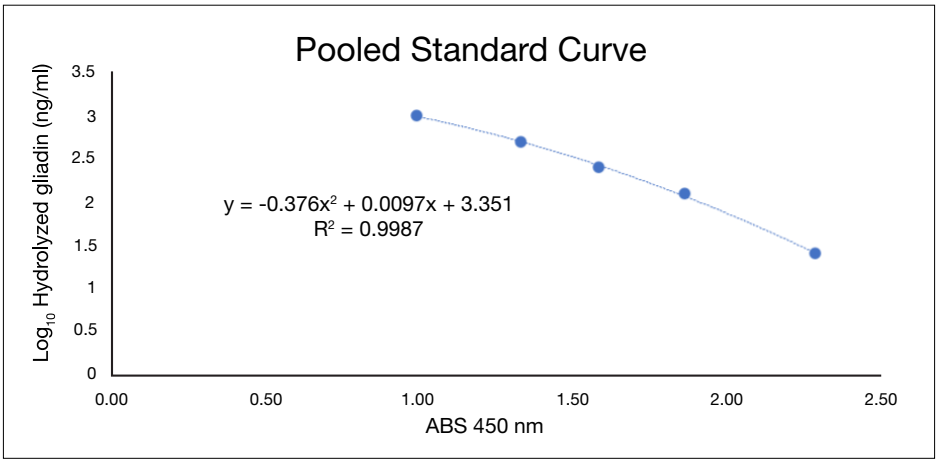

Figure 1. Representation of a standard curve with pooled data of the 12 replications of the intra-assay variation. Raw data on Table 1.

The coefficient of variation (CV) of the absorbances ranges from 5% to 9% depending on the concentration. The average CV is 7%, within the acceptance criteria that is below 10%. In terms of quantification, the coefficient of variation ranges between 2% to 7% with an average CV of 5%.

B) Inter-Assay Variation

The inter-assay variation was determined by testing twelve different test runs of the same kit lot. Tables 3 (absorbances) and 4 (quantifications) and Figure 2 summarize the results of the intra-assay experiments.

Table 3: Analytical inter-assay variation of GlutenTox® ELISA Competitive G12. Absorbance values at 450 nm.

Hydrolyzed gliadin (ng/ml)	Stdr.	1	2	3	4	5	6	7	8	9	10	11	12	Average	SD	%CV
1000	S1	0.99	0.95	0.99	0.93	1.00	0.98	0.91	0.92	0.91	0.93	0.93	0.91	0.95	0.04	4%
500	S2	1.29	1.24	1.27	1.23	1.31	1.32	1.23	1.19	1.34	1.23	1.21	1.28	1.26	0.05	4%
250	S3	1.55	1.54	1.61	1.54	1.71	1.64	1.41	1.58	1.56	1.62	1.54	1.57	1.57	0.07	5%
125	S4	1.77	1.81	1.91	1.77	1.87	1.99	1.75	1.77	1.78	1.81	1.81	1.79	1.82	0.07	4%
25	S5	2.20	2.21	2.24	2.32	2.27	2.29	2.08	2.20	2.11	2.17	2.14	2.24	2.20	0.07	3%
2000	C +	0.61	0.65	0.61	0.61	0.64	0.67	0.57	0.62	0.61	0.62	0.63	0.64	0.62	0.02	4%
0	C -	2.43	2.39	2.54	2.52	2.60	2.59	2.50	2.47	2.58	2.61	2.50	2.50	2.52	0.07	3%
															Average	**4%**

Table 4: Analytical inter-assay variation of GlutenTox® ELISA Competitive G12. Quantifications (ng/ml hydrolyzed gliadin).

Hydrolyzed gliadin (ng/ml)	Stdr.	1	2	3	4	5	6	7	8	9	10	11	12	Average	SD	%CV	% Recovery
1000	S1	994	955	909	995	941	922	963	930	999	918	925	993	954	34.10	4%	95%
500	S2	509	542	588	513	581	568	493	584	490	603	577	512	547	41.34	8%	109%
250	S3	247	257	262	236	217	279	303	224	268	226	256	244	252	24.72	10%	101%
125	S4	125	113	101	130	130	93	101	126	118	119	106	126	116	12.58	11%	93%
25	S5	25	26	27	25	25	29	27	25	25	26	27	25	26	1.17	4%	104%
															Average	**7%**	**100%**

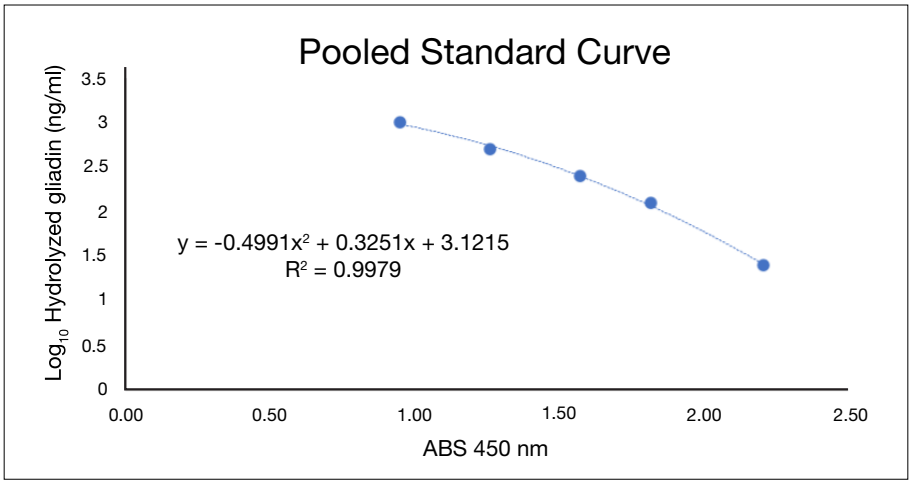

Figure 2. Representation of a standard curve with pooled data of the 12 experiments of the inter-assay variation. Raw data on Table 3.

The coefficient of variation of the absorbances ranges from 3% to 5% depending on the concentration of the standard. The average CV is 4%, within the acceptance criteria that is below 10%. In terms of quantification, the coefficient of variation ranges between 4% to 11% with an average CV of 7%. The correct standard quantification demonstrates the detection accuracy, ensuring accurate sample quantification. The recovery of the standards is between 93% and 109% with an average recovery of 100%.

C) Uncertainty

Uncertainty is calculated using the CVs obtained for each standard in the accuracy and precision assays. The expanded uncertainty ($U_{expanded}$) is calculated to cover 95% of results obtained with the assay. This means that 95 out of 100 results from this assay will be covered by the value of the expanded uncertainty. We defined the maximum expanded uncertainty for our assay to be 35%. The maximum value for the $U_{expanded}$ in this assay is 18.9%. Table 5 and Figure 3 summarize the results of these experiments.

Table 5: Uncertainty of the assay

Hydrolyzed gliadin (ng/ml)	$U_{accuracy}$	$U_{precision}$	U_{total}	$U_{expanded}$
1000	8.7	3.7	9.5	18.9
500	7.6	3.8	8.5	17.0
250	6.0	4.5	7.5	15.1
125	6.6	3.9	7.7	15.4
25	5.2	3.2	6.1	12.3
Total	**6.8**	**3.8**	**7.8**	**15.7**

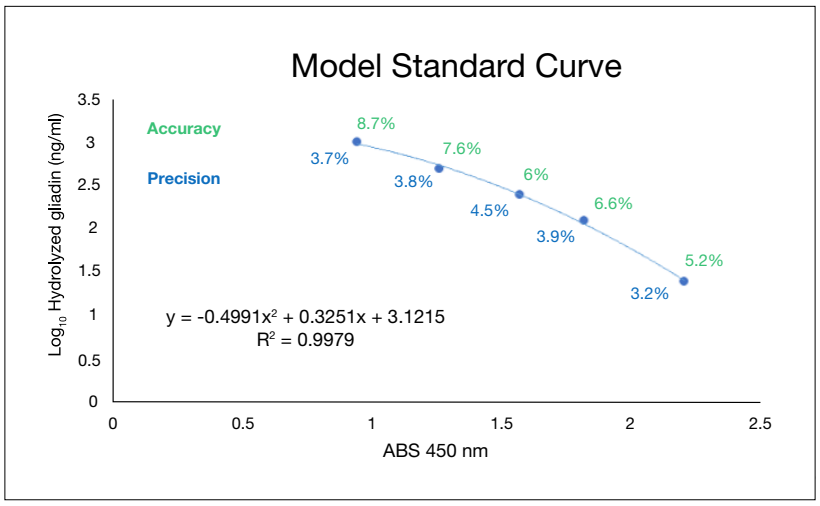

Figure 3. Representative standard curve of the assay with the Accuracy and Precision CVs

D) Linearity

Since the standard values fit to a polynomial function, the analysis of linearity was done using the method of mobile slopes (MS) between standard values and the regression coefficient R^2. Mobile slopes between datasets are calculated as in formula [1].

$$[1]\ MS = (Ym+1 - Ym) / (Xm+1 - Xm)$$

Where m is the order number of the point in the regression line. The CV between the different mobile slopes among different datapoints will be calculated and must be <20%. The regression coefficient is calculated from the polynomial function obtained with the standard curve. This value should be >0.99. Table 6, Table 7, and Figure 4 summarize the results of these experiments.

Table 6: Mobile slopes (MS) analysis

		S1-S2			S2-S3			S3-S4			S4-S5		
Standard Curve	R^2	1000	500	MS	500	250	MS	250	125	MS	125	25	MS
1	0.9999	0.99	1.29	-1686	1.29	1.55	-949	1.55	1.77	-577	1.77	2.20	-231
2	0.9975	0.95	1.24	-1748	1.24	1.54	-842	1.54	1.81	-460	1.81	2.21	-246
3	0.9930	0.93	1.23	-1698	1.23	1.62	-636	1.62	1.81	-665	1.81	2.17	-279
4	0.9927	0.93	1.21	-1751	1.21	1.54	-768	1.54	1.81	-470	1.81	2.14	-300
5	0.9998	0.91	1.28	-1377	1.28	1.57	-838	1.57	1.79	-566	1.79	2.24	-227

Table 7: %CV for curves analyzed in Table 6

MS	Average	SD	%CV
S1-S2	-1652	156	9%
S2-S3	-806	115	14%
S3-S4	-548	85	15%
S4-S5	-257	32	12%
		Average	**13%**

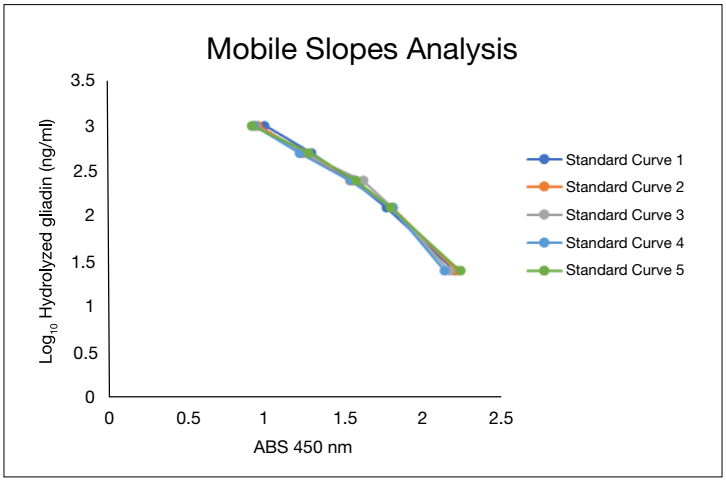

Figure 4. Graphic representation of the 5 standard curves analyzed in Table 6.

The analytical validation shows very good values for precision and accuracy, parameters that define a reproducible and reliable assay. The linearity analysis demonstrates that the standards have been defined correctly and that they will provide results that are inversely proportional to the concentration of the analyte in the test sample.

3. Matrix Analysis (Functional Validation)

Three food matrices were chosen for this validation. Matrices were selected to cover the most common food and drink types: beer, baby food and soy sauce.

A) Accuracy

The accuracy was determined analyzing four different samples of each blank matrix spiked at four different levels of gluten in the analytical range of the kit (5, 10, 20 and 50 ppm gluten). Each sample was analyzed in 8 replicates within the same assay. Results are summarized in Table 8. CV values range from 2% to 5%.

Table 8: Matrix analysis
Accuracy of GlutenTox® ELISA Competitive G12

Matrix	Spiking level (ppm gluten)	Result (ppm gluten) Avg. of n=8	SD	%CV
Beer	0	< LOQ	–	–
	5	3.99	0.04	2%
	10	6.43	0.05	2%
	20	14.51	0.03	2%
	50	32.18	0.03	2%
Baby food	0	< LOQ	–	–
	5	2.25	0.09	4%
	10	6.50	0.03	2%
	20	8.57	0.10	5%
	50	34.81	0.04	3%

B) Precision

The precision was determined by analyzing samples of the two matrices described above, spiked at four different levels of gluten in the analytical range of the kit (5, 10, 20 and 50 ppm gluten). For this determination, three different extractions were analyzed of each spiking level in three different assays, each assay on a different day. Each analysis had 4 replicates of each sample. Results are summarized in Table 9.
CV values range between 11% and 17%.

Table 9: Matrix analysis
Precision of GlutenTox® ELISA Competitive G12

Matrix	Spiking level (ppm gluten)	Result (ppm gluten) Avg. of n=12	SD	%CV
Beer	0	< LOQ	–	–
	5	3.96	0.43	11%
	10	8.08	1.21	15%
	20	17.37	2.36	14%
	50	39.93	4.82	12%
Baby food	0	< LOQ	–	–
	5	4.14	0.69	17%
	10	7.13	0.98	14%
	20	11.76	1.81	15%
	50	36.23	5.28	15%

C) Uncertainty

Functional uncertainty of the assay was calculated with the CVs obtained in the Accuracy and Precision experiments. The expanded uncertainty ($U_{expanded}$) is calculated to cover 95% of results obtained with the assay. This means that 95 out of 100 results from this assay will be covered by the value of the expanded uncertainty. We defined the maximum expanded uncertainty to be 35% based on our standard acceptance criteria. Results are summarized in Table 10. The expanded uncertainty in all samples tested is below 35%, with the highest value obtained being 34.6%.

Table 10: Functional uncertainties of GlutenTox® ELISA Competitive G12

Matrix	Spiking level (ppm gluten)	$U_{repeatibility}$	$U_{reproducibility}$	U_{total}	$U_{expanded}$
Beer	5	1.98	10.96	11.14	22.27
	10	2.39	15.01	15.20	30.40
	20	1.55	13.59	13.68	27.36
	50	1.83	12.08	12.22	24.44
Baby food	5	4.27	16.76	17.30	34.60
	10	1.55	13.76	13.84	27.69
	20	5.42	15.37	16.29	32.59
	50	2.99	14.58	14.88	29.76

D) Recovery

To test for recovery, the same blank matrices were spiked with hydrolyzed gliadin in the analytical range of the kit (5, 10, 20 and 50 ppm gluten).

Spiked samples were extracted following manual instructions and assayed. Results of the recovery experiments are summarized in Tables 11 and 12. Average recovery for three matrices ranges from 68% to 92%.

Table 11: Matrix analysis Recovery

Matrix	Spiking level (ppm gluten)	%Recovery
Beer	5	57%
	10	72%
	20	92%
	50	93%
Soy Sauce	5	80%
	10	84%
	20	92%
	50	114%
Baby food	5	62%
	10	75%
	20	55%
	50	79%

Table 12: Matrix analysis
Average recovery by matrix

Matrix	%Recovery
Beer	79%
Soy Sauce	92%
Baby food	68%

4. Analytical Sensitivity

To determine the analytical sensitivity, Dilution Solution was assayed 24 times in the inter- and intra- assay experiment. Mean response of blank ($\overline{B}$) in ng/ml and standard deviation was calculated. The corresponding concentration of $\overline{B}$ + 3x standard deviation was defined as limit of detection. Results are summarized in Table 13.

Table 13: Analytical sensitivity

Blank replicates (ng/ml)					
Curve 1	0.80	9.36	Curve 7	6.67	3.70
Curve 2	3.86	12.39	Curve 8	7.85	7.39
Curve 3	7.81	6.43	Curve 9	11.34	1.38
Curve 4	7.45	12.47	Curve 10	6.00	2.25
Curve 5	3.61	5.14	Curve 11	5.77	4.14
Curve 6	10.29	6.83	Curve 12	6.27	7.89
Average			**6.55**		
SD			**3.17**		
				ng/ml	ppm
Limit of Detection (LoD)				**16.07**	**1.61**

The limit of detection (LoD) calculated for the kit is 1.6 ppm of gluten. The limit of quantification (LoQ) is defined by the lowest standard of the curve, and it is 2.5 ppm of gluten.

5. Fapas Proficiency Table

Table 14: FAPAS proficiency testing Dec 2021

Type of matrix	Identification	Hygiena™ Lab number	Result mg/kg for Lab 035 (Hygiena™)
Gluten in beer	27308A	035	7.16
Gluten in beer	27308B	035	Not detected (below LOQ)
Z-score for Lab 035 (Hygiena™)	**Assigned value mg/kg**	**Assessment for Lab 035 (Hygiena™)**	**Number of participants who received samples**
-1.65	12.2	Satisfactory	42 (100%)
Qualitative	92% not detected (negative)	Agreed	42 (100%)
Number of participants submitting results	**Total number of results statistically analyzed**	**Range of results from other labs using same kit (assigned value)**	**Number of results with |z| ≤2 included in assigned value**
38 (90%)	28	ND to 28,7 ppm	21 (75%)
38 (90%)	Qualitative	ND (92%)	Qualitative

6. Discussion

The analytical validation shows a standard curve design that has accuracy and precision values below 10% CV. The expanded uncertainty for the standard values is below 20%. The results show that the standard curve will provide reliable results for sample quantification in terms of linearity, repeatability, and reproducibility.

The results for precision and accuracy between the matrices assayed (2 matrices) stayed below 10% CV for accuracy and below 20% CV for precision. The average recovery of all matrices assayed (3 matrices) ranged from 68% to 92%.

7. Conclusion

GlutenTox® ELISA Competitive G12 is a new-generation kit for hydrolyzed gluten determination in foods and drinks. It uses the monoclonal antibody G12 raised specifically against the 33-mer within the α-gliadin molecule, which has been described as the most immunotoxic fraction of gluten. The 33-mer is the peptide that causes most immunotoxicity (and therefore symptoms) in celiac patients. GlutenTox® ELISA Competitive G12 is an ideal method to detect the presence of hydrolyzed gluten in beer after fermentation. Proficiency testing of beer samples shows that it can correctly quantify hydrolized gluten in beer and discriminate beer samples that are exempt of gluten.

8. References

[1] MORÓN B., et al., “Toward the Assessment of Food Toxicity for Celiac Patients: Characterization of Monoclonal Antibodies to a Main Immunogenic Gluten Peptide”; 2008; PLoS 0NE 3: e2294.3.

[2] SHAN L., et al.; “Structural basis for gluten intolerance in celiac sprue”; Science; 2002; 297: 2275-9.

[3] AOAC International. “Guidelines for Standard Methods Performance Requirements”. 2016