Validation Report

GlutenTox® ELISA Rapid G12

KIT3075

INDEX

1. Scope and description of the product ... 2
2. Analytical Validation ... 2
   a) Intra-Assay Variation ... 2
   b) Inter-Assay Variation ... 3
   c) Uncertainty ... 4
   d) Linearity ... 5
3. Hook Effect ... 6
4. Stability ... 8
   a) Stability of GlutenTox A1-HRP Antibody ... 8
   b) Stability of GlutenTox ready-to-use Standards ... 10
5. Matrix Analysis (functional validation) ... 12
   a) Accuracy ... 12
   b) Precision ... 14
   c) Uncertainty ... 16
   d) Recovery ... 18
6. Surface Validation ... 19
7. Analytical Sensitivity ... 20
8. Cross-Reactivity ... 21
9. Robustness ... 22
10. Discussion ... 25
11. Conclusion ... 26
12. References ... 27

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1. Scope and description of the product

GlutenTox ELISA Rapid G12 is an immunosorbent assay for the determination of the immunotoxic fraction of gluten that is harmful to celiac patients. The present report describes the validation process and its results.

GlutenTox ELISA Rapid G12 is based on the G12 and A1 antibodies, which specifically recognize 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 (50, 25, 12.5, 6.25 and 1.56 ng/mL gliadin). Table 1 (absorbances) and Table 2 (quantifications) and Figure 1 summarize the results of the intra-assay experiments.

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

ng/mL gliadin	Std	1	2	3	4	5	6	7	8	9	10	11	12	Avg.	SD	CV%
100	C+	2.65	2.56	2.65	2.69	2.72	2.69	2.69	2.80	2.80	2.82	2.90	2.99	2.75	0.12	4%
50	Std1	1.67	1.66	1.68	1.69	1.71	1.72	1.75	1.74	1.80	1.87	1.89	2.02	1.77	0.11	6%
25	Std 2	0.92	0.90	0.93	0.94	0.95	0.98	0.99	0.99	1.01	1.00	1.03	1.14	0.98	0.06	7%
12.5	Std 3	0.51	0.51	0.51	0.50	0.52	0.53	0.54	0.53	0.54	0.56	0.56	0.65	0.54	0.04	7%
6.25	Std 4	0.28	0.28	0.29	0.28	0.29	0.30	0.31	0.30	0.28	0.30	0.62*	0.37	0.30	0.03	9%
1.56	Std 5	0.12	0.11	0.11	0.11	0.12	0.11	0.12	0.12	0.12	0.11	0.12	0.14	0.12	0.01	7%
	C-	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.07	0.06	0.00	6%
Average																**7%**

**Documented error by the operator. Not used in statistical analysis.*

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Table 2. Analytical intra-assay variation of GlutenTox ELISA Rapid G12. Quantifications (ng/mL gliadin).

ng/mL gliadin	Std	1	2	3	4	5	6	7	8	9	10	11	12	Avg.	SD	CV %	% Recovery
100	**C+**	**82.25**	**78.57**	**82.09**	**83.73**	**84.91**	**83.89**	**83.89**	**88.04**	**88.12**	**88.92**	**92.30**	**95.79**	**83.42**	**2.69**	**3%**	**83%**
50	Std1	46.61	46.31	47.18	47.28	48.12	48.46	49.58	49.20	51.28	53.45	54.14	58.66	47.84	1.19	2%	96%
25	Std 2	23.32	22.71	23.47	23.73	24.08	24.93	25.26	25.23	25.79	25.73	26.36	29.85	24.09	0.96	4%	96%
12.5	Std 3	11.72	11.69	11.80	11.58	11.91	12.42	12.53	12.28	12.50	13.04	13.18	15.56	11.99	0.36	3%	96%
6.25	Std 4	5.69	5.77	5.87	5.69	5.92	6.23	6.41	6.18	5.69	6.13		8.07	5.97	0.27	5%	96%
1.56	Std 5	1.61	1.41	1.58	1.46	1.61	1.56	1.61	1.61	1.63	1.53	1.73	2.17	1.55	0.08	5%	99%
Average																4%	94%

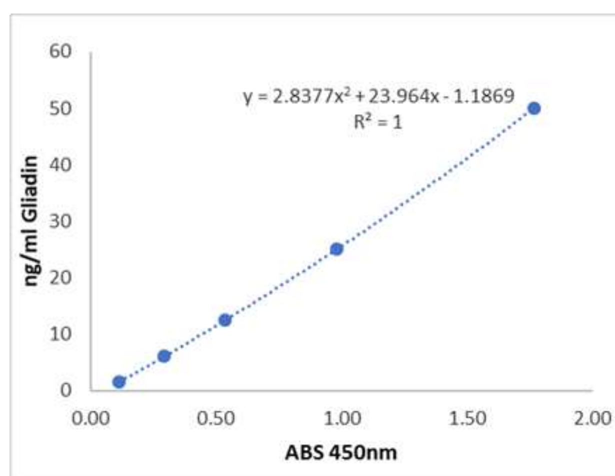

Figure 1. Representation of a standard curve with pooled data of the 12 repetitions of the intra-assay variation. Raw data on Table 1.

The coefficient of variation (CV) of the absorbances ranges from 4% to 9% depending on the concentration. The average CV is 7%, below the 10% of acceptance criteria. In terms of quantification, the coefficient of variation ranges between 2%-5% with an average CV of 4%.

b) Inter-Assay Variation

The inter-assay variation was determined by testing twelve different test runs of the same kit lot. Results of these experiments are shown in Figure 2.

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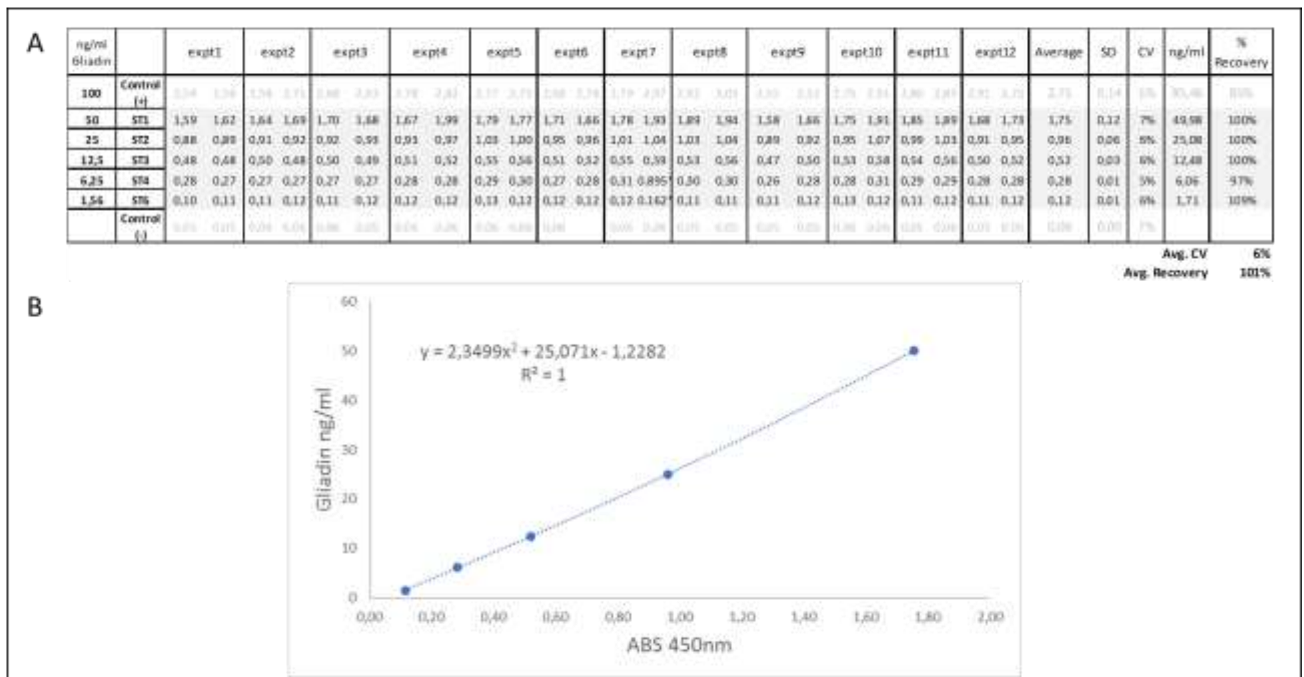

A

ng/ml Gliadin		expt1		expt2		expt3		expt4		expt5		expt6		expt7		expt8		expt9		expt10		expt11		expt12		Average	SD	CV	ng/ml	% Recovery
100	Control (+)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
50	ST1	1,59	1,62	1,64	1,69	1,70	1,68	1,67	1,99	1,79	1,77	1,71	1,66	1,78	1,93	1,89	1,94	1,58	1,66	1,75	1,91	1,85	1,89	1,68	1,73	1,75	0,12	7%	49,98	100%
25	ST2	0,88	0,89	0,91	0,92	0,92	0,93	0,93	0,97	1,03	1,00	0,95	0,96	1,01	1,04	1,03	1,04	0,89	0,92	0,95	1,07	0,99	1,03	0,91	0,95	0,96	0,06	6%	25,08	100%
12,5	ST3	0,48	0,48	0,50	0,48	0,50	0,49	0,51	0,52	0,55	0,56	0,51	0,52	0,55	0,59	0,53	0,56	0,47	0,50	0,53	0,58	0,54	0,56	0,50	0,52	0,53	0,03	6%	12,48	100%
6,25	ST4	0,28	0,27	0,27	0,27	0,27	0,27	0,28	0,28	0,29	0,30	0,27	0,28	0,31	0,895*	0,30	0,30	0,26	0,28	0,28	0,31	0,29	0,29	0,28	0,28	0,28	0,01	5%	6,06	97%
1,56	ST6	0,10	0,11	0,11	0,12	0,11	0,12	0,12	0,12	0,13	0,12	0,12	0,12	0,12	0,162*	0,11	0,11	0,11	0,12	0,13	0,12	0,11	0,12	0,11	0,12	0,12	0,01	6%	1,71	109%
	Control (-)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		

Avg. CV 6%
Avg. Recovery 101%

B

Figure 2. Results of the experiments to determine the inter-assay variation of GlutenTox ELISA Rapid G12. (A) Absorbances, quantification in ng/mL gliadin of the Standards and percentage of recovery. (B) Representative curve built with the pooled data of the 12 experiments.

The coefficient of variation ranges from 5% to 7% depending on the concentration of the standard. The average CV is 6%, below the 10% of acceptance criteria. The power of quantification of the function is assessed by quantifying the standards; the recovery of the standards is between 97% and 109% with an average recovery of 101%.

c) Uncertainty

Uncertainty is calculated using the CVs obtained for each standard in the accuracy and precision assays. The expanded uncertainty (U_{exp}) 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_{exp} in this assay is 16.1%. Figure 3 and **Table 3** summarize the results of these experiments.

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Accuracy
Precision

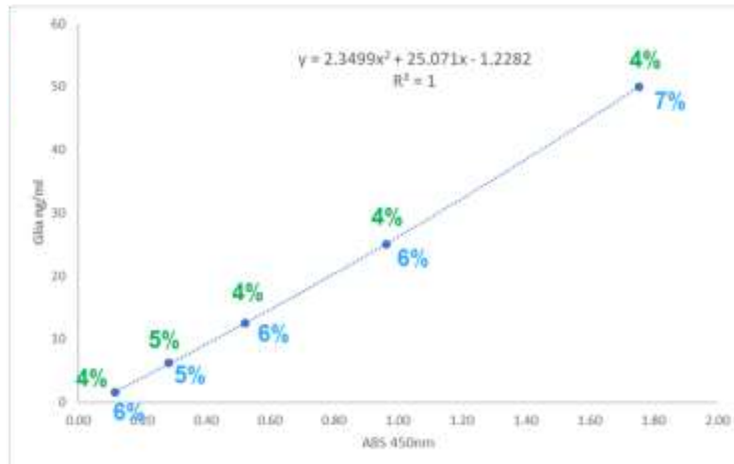

Figure 3. Representative standard curve of the assay with the Accuracy and Precision CVs.

Table 3. Uncertainty of the assay

	Accuracy	Precision		
ng/mL gliadin	U_{acc}	U_{prec}	U_{total}	$U_{expanded}$
50	4.00	7.0	8.06	16.12
25	4.00	6.0	7.21	14.42
12.5	4.00	6.0	7.21	14.42
6.25	5.00	5.0	7.07	14.14
1.56	4.00	6.0	10.63	21.26
Total	4.20	6.0	7.21	14.42

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 calculated as in formula [1].

$$[1]\ MS = (Y_{m+1} - Y_m) / (X_{m+1} - X_m)$$

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%. Regression coefficient: Calculated from the polynomial function obtained with the standard curve. This value should be >0.99. Figure 4 summarizes the results of these experiments.

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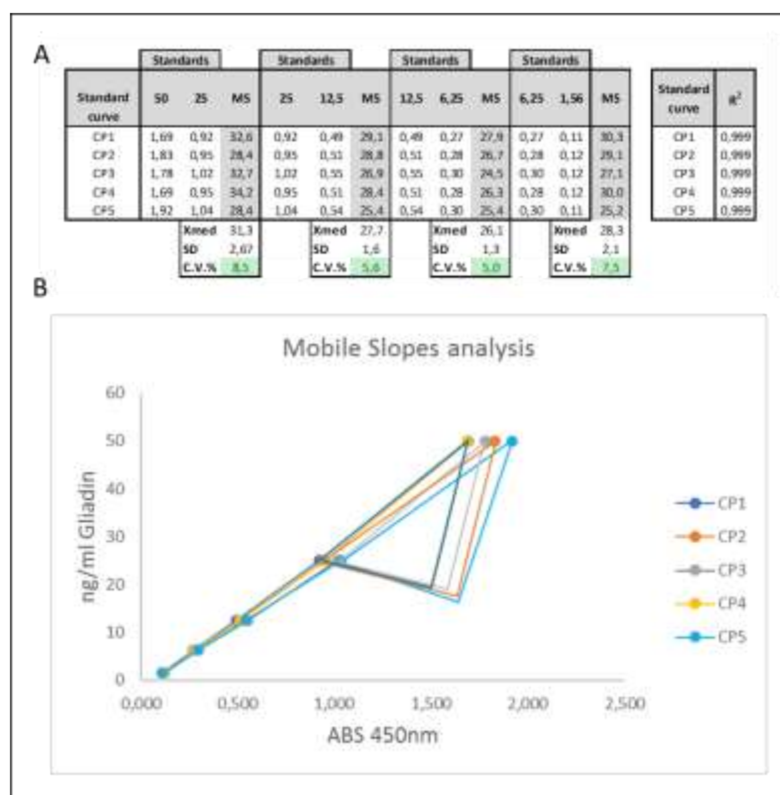

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Standard curve	Standards 50	25	MS	Standards 25	12,5	MS	Standards 12,5	6,25	MS	Standards 6,25	1,56	MS
CP1	1,69	0,92	32,6	0,92	0,49	29,1	0,49	0,27	27,9	0,27	0,11	30,3
CP2	1,83	0,95	28,4	0,95	0,51	28,8	0,51	0,28	26,7	0,28	0,12	29,1
CP3	1,78	1,02	32,7	1,02	0,55	26,9	0,55	0,30	24,5	0,30	0,12	27,1
CP4	1,69	0,95	34,2	0,95	0,51	28,4	0,51	0,28	26,3	0,28	0,12	30,0
CP5	1,92	1,04	28,4	1,04	0,54	25,4	0,54	0,30	25,4	0,30	0,11	25,2
		Xmed	31,3		Xmed	27,7		Xmed	26,1		Xmed	28,3
		SD	2,67		SD	1,6		SD	1,3		SD	2,1
		C.V.%	8,5		C.V.%	5,8		C.V.%	5,0		C.V.%	7,5

Standard curve	R^2
CP1	0,999
CP2	0,999
CP3	0,999
CP4	0,999
CP5	0,999

Figure 4. (A) Mobile slopes (MS) analysis, R^2 and CVs for 5 different standard curves. (B) Graphic representation of the 5 standard curves analyzed in (A).

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

3. Hook effect

The hook effect or the prozone effect is an immunological phenomenon whereby the effectiveness of antibodies to form immune complexes is sometimes impaired when concentrations of an antibody or an antigen is very high. The formation of immune complexes stops increasing with greater concentrations of analyte and then decreases with extremely high concentrations thereof, producing a hook shape on a graph of measurements. To study this effect, a pure wheat flour extract was analyzed at different dilutions. All results were positive. The quantitative capacity of the test at high dilution is also verified in this assay where dilutions such as 1:50000, 1:100000, 1:250000 and 1:500000 provided very similar quantification values, with an average CV of 4%. We conclude that GlutenTox ELISA Rapid G12 shows no hook effect. Tables 4 and 5 summarize the results of these experiments.

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Table 4. Analysis of hook effect in GlutenTox ELISA Rapid G12.

Dilution (1:X)	Sample	ABS 450 nm		Avg.	SD	CV%	Gliadin (ng/mL)	Gluten (ppm)
1	Wheat flour	(+)	(+)	>ULoQ			>ULoQ	
2		(+)	(+)	>ULoQ			>ULoQ	
5		(+)	(+)	>ULoQ			>ULoQ	
10		(+)	(+)	>ULoQ			>ULoQ	
20		(+)	(+)	>ULoQ			>ULoQ	
50		(+)	(+)	>ULoQ			>ULoQ	
100		(+)	(+)	>ULoQ			>ULoQ	
250		(+)	(+)	>ULoQ			>ULoQ	
500		(+)	(+)	>ULoQ			>ULoQ	
1000		(+)	(+)	>ULoQ			>ULoQ	
5000		(+)	(+)	>ULoQ			>ULoQ	
10000		(+)	(+)	>ULoQ			>ULoQ	
50000		3.081	2.905	2.993	0.124	4%	96.33	96326.33
100000		1.867	1.99	1.929	0.087	5%	50.50	101001.10
250000		1.025	0.941	0.983	0.059	6%	20.47	102356.65
500000		0.529	0.517	0.523	0.008	2%	9.49	94928.32

(+): saturated; ULoQ: Upper limit of quantification

Table 5. Quantification of wheat flour with GlutenTox ELISA Rapid G12.

Dilution (1:X)	Gluten (ppm)	Average	SD	%CV
50000	96326.33	98653.1	358.3	4%
100000	101001.10			
250000	102356.65			
500000	94928.32			

It is of great importance that a food analysis kit does not show hook effect, so it will always be positive in the presence of the analyte (i.e. does not show false negatives). Analyzing samples with wheat flour as ingredient poses a problem to those kits that present hook effect. In contrast, the GlutenTox ELISA Rapid G12 is able to quantify

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4. Stability

The critical components of the kit were subjected to a real-time and an accelerated stability study. GlutenTox A1-HRP antibody and GlutenTox ready-to-use Standards were placed at 4 °C / 39 °F, room temperature (15-25 °C / 59-77 °F) and 42 °C /107 °F for various weeks/months.

a) Stability of GlutenTox A1-HRP antibody.

One batch of antibody was prepared and divided into the three temperatures. For each timepoint, the OD obtained by the study antibody was compared with the OD obtained with a fresh antibody prepared the same day in an assay that contained three standards of the standard curve (50, 12.5 and 1.56 ng/mL gliadin).

The real time stability study has been conducted for 16 months at 4 °C / 39 °F (the recommended storage temperature). GlutenTox A1-HRP antibody shows a performance within 80% and 120% in all cases (Figure 5).

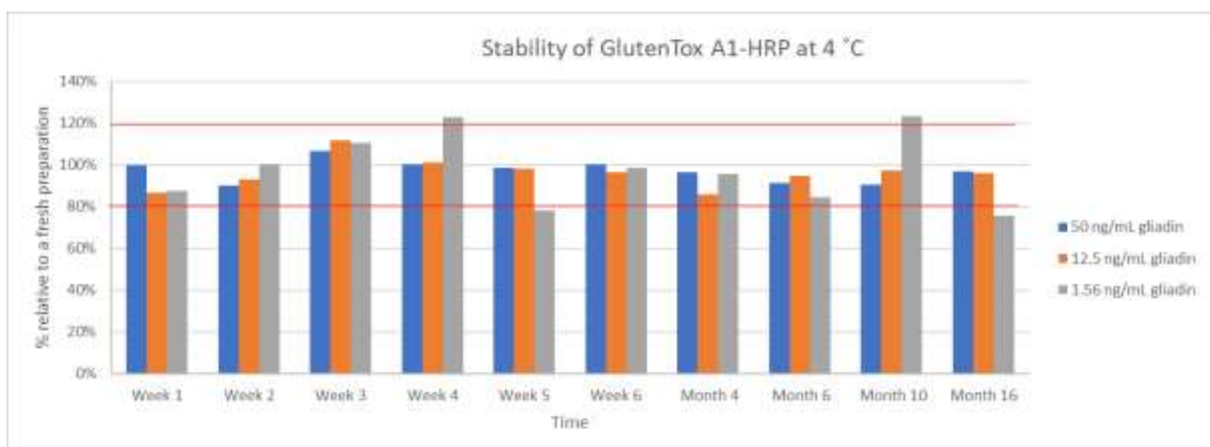

Figure 5. Real-time stability study of A1-HRP antibody at 4 °C / 39 °F.

The accelerated stability study was performed by subjecting the antibody to stress temperatures: 15-25 °C / 59-77 °F and 42 °C /107 °F. In the most extreme temperature (42 °C /107 °F), the antibody shows a decay in the first week of two of the three tested standards down to 60% of its performance compared to a fresh preparation. However, this lower activity is maintained -for the three standards- for a total of 6 weeks, showing a relative stability after a considerable decay (Figure 6).

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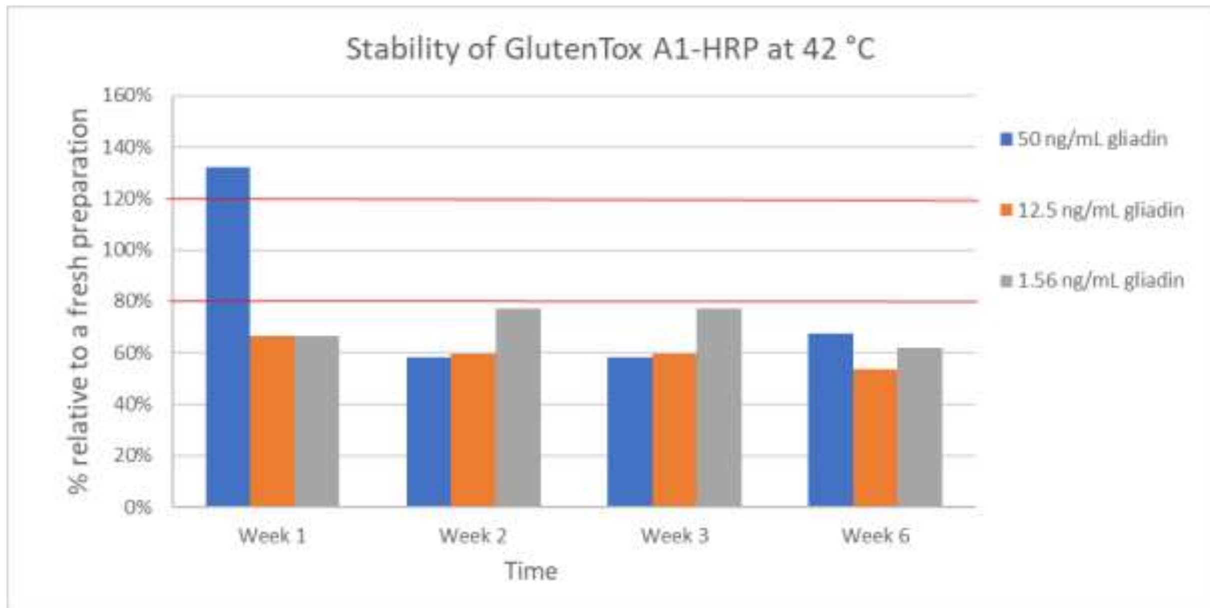

Figure 6. Accelerated stability study of A1-HRP antibody at 42 °C /107 °F.

A second accelerated stability study was performed at room temperature (15-25 °C / 59-77 °F). In this case, the antibody maintains an acceptable activity (>80%) for 6 weeks (Figure 7).

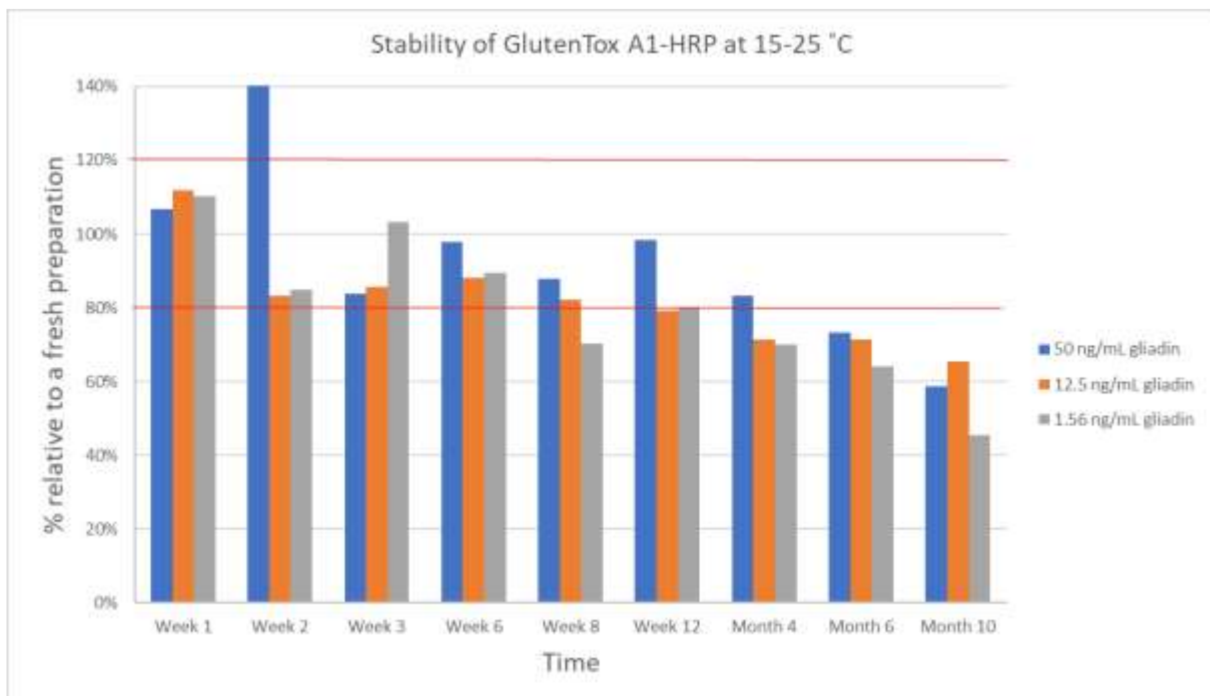

Figure 7. Accelerated stability study of A1-HRP antibody at 15-25 °C / 59-77 °F.

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These results show that GlutenTox A1-HRP antibody is stable over long periods of time and will withstand temperature swings caused by accident, transportation or human mistakes in storage.

b) Stability of the GlutenTox ready-to-use Standards.

Similar stability studies were conducted to test the stability of the ready-to-use standards included in the kit. Three different batches (A, B and C) were prepared of the whole standard curve (Positive Control, 50, 25, 12.5, 6.25, 1.56 ng/mL gliadin and Negative Control) and subjected to different temperatures: 4 °C / 39 °F, room temperature (15-25 °C / 59-77 °F) and 42 °C /107 °F. In each timepoint (see **Table 6**) the three batches of each temperature were tested against a freshly prepared Standard curve and normalized to it, to account for inter-assay variations.

Table 6. Timepoints of GlutenTox Standards stability study

Date	Time
T1	1 Week
T2	2 Weeks
T3	3 Weeks
T4	4 Weeks
T5	5 Weeks
T6	6 Weeks
T7	2 months
T8	3 months
T9	6 months

To analyze the data, we selected three of the 5 Standards to show their stability. However, the rest of the curve shows a similar behavior (data not shown).

The real-time stability study was performed at 4 °C / 39 °F, the recommended storage temperature. Figure 8 shows the OD obtained of the three standards of each batch in each timepoint. The horizontal colored lines represent ± 20% of the average OD of all datapoints. The results show that the curve maintains a good activity for 6 months (study ongoing).

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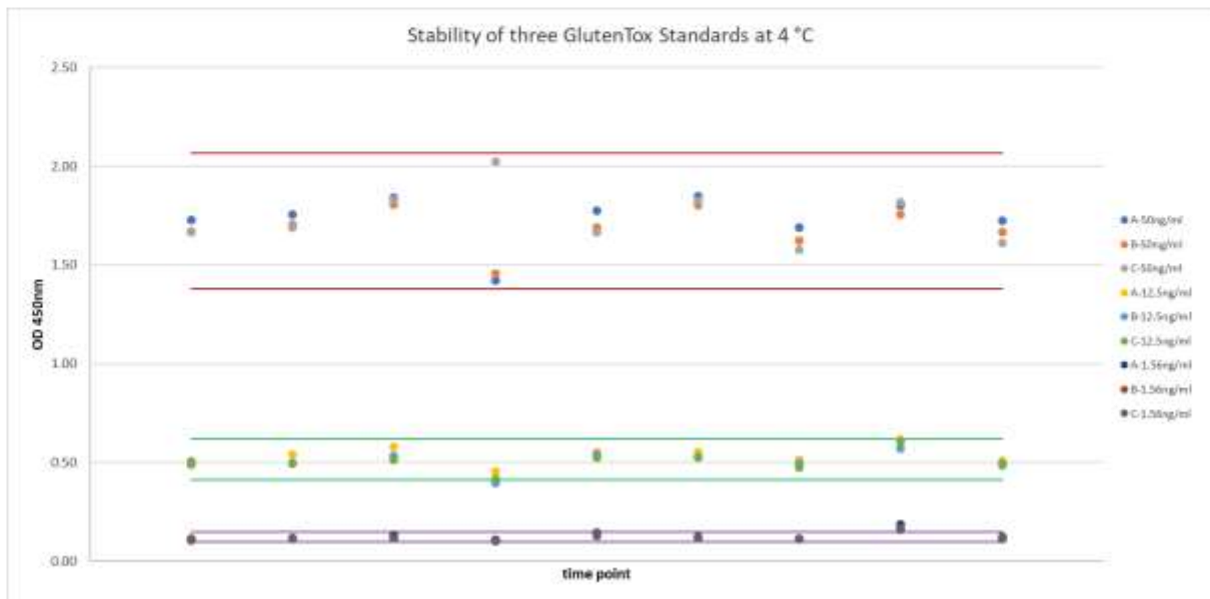

Figure 8. Results of stability study of GlutenTox Standards at 4 °C / 39 °F.

The accelerated stability studies were conducted at room temperature (15-25 °C / 59-77 °F) and 42 °C /107 °F. The results at room temperature show similar results as the results obtained when stored cold. The activity is maintained for 6 months with no change (see Figure 9).

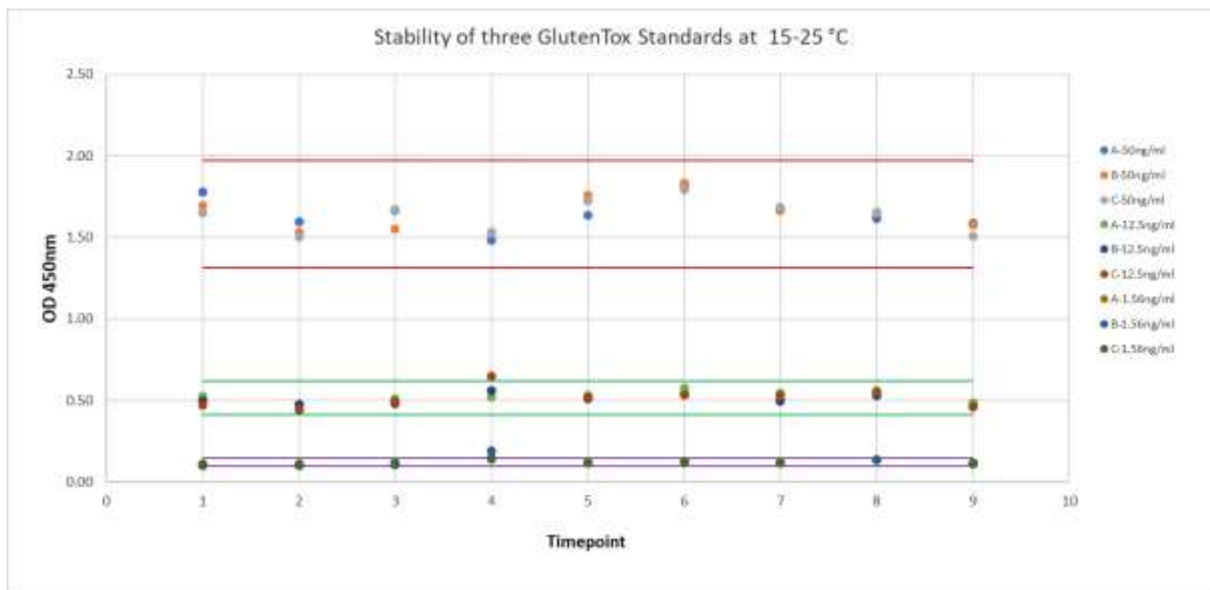

Figure 9. Results of stability study of GlutenTox Standards at room temperature.

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Similar results although with a higher variation between datapoints are obtained at 42 °C /107 °F. Results are shown in Figure 10.

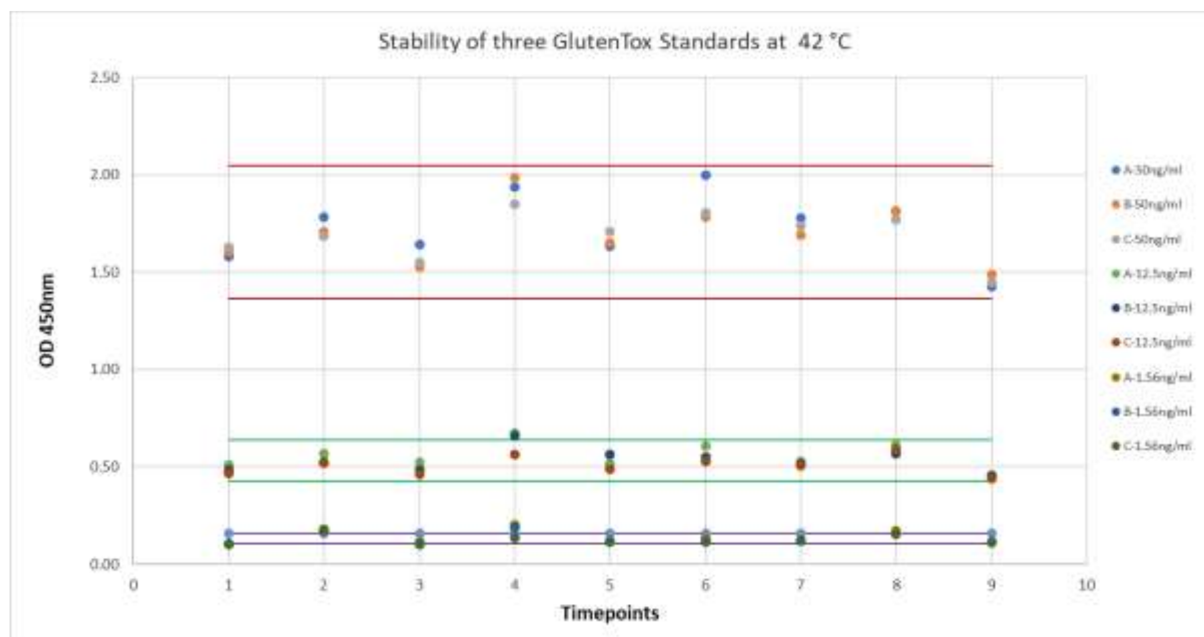

Figure 10. Results of stability study of GlutenTox Standards at 42 °C /107 °F.

The accelerated stability studies results show that the ready-to-use gliadin Standards are stable for at least 6 months in extreme temperatures, thus conferring the Standards with a great stability in optimal conditions. They will withstand temperature shifts due to transportation, inappropriate accidental storage or human mistakes.

5. Matrix Analysis (functional validation)

In total, twelve food matrices were chosen for this validation. Matrices were selected to cover the most commonly used food types: baked goods (bread, biscuits, rice crackers), soy products (soy flour, ground soybeans, soy sauce), dairy products (yogurt), chocolate, meat (pâté) and others (soup, rolled oats and a mix of spices).

a) Accuracy

The accuracy was determined analyzing three different samples of each blank matrix spiked at three different levels of gluten in the analytical range of the kit (5, 20 and 40 ppm gluten). Each sample was analyzed in 8 replicates within the same assay. Results are summarized in **Table 7**. CV values range from 1% to 9%.

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Table 7. Matrix analysis- Accuracy of GlutenTox ELISA Rapid G12					
Group	**Matrix**	**Spiking level (ppm gluten)**	**Result (ppm gluten, avg. of n=8)**	**SD**	**CV%**
Baked goods	**Bread**	**5**	6.09	0.24	4%
		20	21.05	0.85	4%
		40	29.50	2.13	7%
	Biscuits	**5**	4.60	0.17	4%
		20	20.34	1.38	7%
		40	39.03	2.18	6%
	Rice Crackers	**5**	5.13	0.17	3%
		20	20.38	0.82	4%
		40	39.72	1.28	3%
Soy products	**Soy flour**	**5**	5.11	0.46	9%
		20	20.16	1.36	7%
		40	42.44	2.00	5%
	Soybeans	**5**	4.56	0.36	8%
		20	18.76	0.86	5%
		40	40.16	1.87	5%
	Soy Sauce	**5**	4.63	0.11	2%
		20	17.55	0.76	4%
		40	32.18	1.98	6%
Dairy products	**Yogurt**	**5**	4.33	0.07	2%
		20	18.00	0.37	2%
		40	31.89	0.91	3%
Chocolate products	**Chocolate**	**5**	3.44	0.14	4%
		20	18.76	1.34	7%
		40	28.41	0.77	3%
Meat	**Pâté**	**5**	5.19	0.29	6%
		20	21.51	1.02	5%
		40	37.82	1.32	3%
Others	**Soup**	**5**	4.87	0.23	5%
		20	17.09	0.77	5%
		40*	35.48	1.12	3%
	Rolled Oats	**5**	5.03	0.29	6%
		20	18.27	0.75	4%
		40	34.29	1.86	5%
	Spice Mix	**5**	4.21	0.21	5%
		20	16.37	0.22	1%
		40	34.59	1.29	4%

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b) Precision

The precision was determined by analyzing samples of the twelve matrices described above, spiked at three different levels of gluten in the analytical range of the kit (5, 20 and 40 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 8**. %CV values range between 3% and 14%.

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Table 8. Matrix analysis- Precision of GlutenTox ELISA Rapid G12

Group	Matrix	Spiking level (ppm gluten)	Result (ppm gluten, avg. of n=12)	SD	CV%
Baked goods	Bread	5	6.29	0.28	4%
		20	22.99	2.45	11%
		40	36.96	5.31	14%
	Biscuits	5	4.76	0.32	7%
		20	19.53	1.14	6%
		40	34.90	2.22	6%
	Rice Crackers	5	6.05	0.71	12%
		20	25.28	3.25	13%
		40	45.10	4.34	10%
Soy products	Soy flour	5	5.43	0.56	10%
		20	21.49	1.46	7%
		40	42.61	2.70	6%
	Soybeans	5	5.05	0.58	12%
		20	19.92	1.68	8%
		40	38.26	2.06	5%
	Soy Sauce	5	5.11	0.53	10%
		20	21.16	2.39	11%
		40	39.40	4.05	10%
Dairy products	Yogurt	5	4.18	0.25	6%
		20	18.60	0.76	4%
		40	31.39	1.19	4%
Chocolate products	Chocolate	5	3.27	0.23	7%
		20	19.38	1.42	7%
		40	34.74	4.87	14%
Meat	Pâté	5	4.59	0.46	10%
		20	19.25	1.03	5%
		40	37.27	4.65	12%
Others	Soup	5	4.82	0.37	8%
		20	18.32	0.63	3%
		40*	34.43	1.74	5%
	Rolled Oats	5	5.20	0.33	6%
		20	19.40	0.81	4%
		40	36.62	2.23	6%
	Spice Mix	5	5.35	0.54	10%
		20	20.26	0.66	3%
		40	38.56	4.67	12%

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c) Uncertainty

Functional uncertainty of the assay was calculated with the CVs obtained in the Accuracy and Precision experiments. The expanded uncertainty (U_{exp}) 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%. Results are summarized in **Table 9**. The expanded uncertainty in all samples tested is below 35%, with the highest value obtained being 31%. The matrices with the lowest uncertainty values (below 20%) are yogurt, soup, biscuits, and rolled oats.

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Table 9. Functional uncertainties of GlutenTox ELISA Rapid G12						
			Accuracy	Precision		
Group	Matrix	Spiking level (ppm gluten)	$U_{repeatibility}$	$U_{repdroducibility}$	U_{total}	$U_{expanded}$
Baked goods	Bread	5	4.00	4.00	5.66	11.31
		20	4.00	11.00	11.70	23.41
		40	7.00	14.00	15.65	31.30
	Biscuits	5	4.0	7.0	8.06	16.12
		20	7.0	6.0	9.22	18.44
		40	6.0	6.0	8.49	16.97
	Rice Crackers	5	3.0	12.0	12.37	24.74
		20	4.0	13.0	13.60	27.20
		40	3.0	10.0	10.44	20.88
Soy products	Soy flour	5	9.0	7.0	11.4	22.8
		20	7.0	6.0	9.2	18.4
		40	5.0	6.0	7.8	15.6
	Soybeans	5	8.0	12.0	14.42	28.84
		20	5.0	8.0	9.43	18.87
		40	5.0	5.0	7.07	14.14
	Soy Sauce	5	2.0	10.0	10.20	20.40
		20	4.0	11.0	11.70	23.41
		40	6.0	10.0	11.66	23.32
Dairy products	Yogurt	5	2.0	6.0	6.32	12.65
		20	2.0	4.0	4.47	8.94
		40	3.0	4.0	5.00	10.00
Chocolate products	Chocolate	5	4.0	7.0	8.06	16.12
		20	7.0	7.0	9.90	19.80
		40	3.0	14.0	14.32	28.64
Meat	Pâté	5	6.0	10.0	11.00	22.00
		20	5.0	5.0	7.07	14.14
		40	3.0	12.0	14.32	28.64
Others	Soup	5	5.0	8.0	9.43	18.87
		20	5.0	3.0	5.83	11.66
		40	3.0	5.0	5.83	11.66
	Rolled Oats	5	6.0	6.0	12.37	24.74
		20	4.0	4.0	8.94	17.89
		40	5.0	6.0	10.44	20.88
	Spice Mix	5	5.0	10.0	11.18	22.36
		20	1.0	3.0	3.16	6.32
		40	4.0	12.0	12.65	25.30

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d) Recovery

To test for recovery, the same blank matrices were spiked with gliadin in the analytical range of the kit (5, 20 and 40 ppm gluten).

Spiked samples were extracted following manual instructions and assayed. Results of the recovery experiments are summarized in Tables 10 and 11. Average recovery for all matrices ranges from 83% to 120%.

Table 10. Matrix analysis-Recovery with GlutenTox ELISA Rapid G12

Group	Matrix	Spiking level (ppm gluten)	% Recovery	Group	Matrix	Spiking level (ppm gluten)	% Recovery
Baked goods	Bread	5	126%	Dairy products	Yogurt	5	84%
		20	115%			20	93%
		40	92%			40	78%
	Biscuits	5	95%	Chocolate products	Chocolate	5	65%
		20	98%			20	97%
		40	87%			40	87%
	Rice Crackers	5	121%	Meat	Pâté	5	92%
		20	126%			20	96%
		40	113%			40	93%
Soy products	Soy Sauce	5	102%	Others	Spice Mix	5	107%
		20	106%			20	101%
		40	98%			40	96%
	Soy flour	5	109%		Soup	5	96%
		20	107%			20	92%
		40	107%			40	86%
	Soybeans	5	101%		Rolled Oats	5	104%
		20	100%			20	97%
		40	96%			40	92%

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Table 11. Matrix analysis-Average recovery by matrix with GlutenTox ELISA Rapid G12		
Group	**Matrix**	**Recovery (%)**
Baked goods	Bread	111%
	Biscuits	93%
	Rice Crackers	120%
Soy products	Soy flour	108%
	Soybeans	99%
	Soy Sauce	102%
Dairy products	Yogurt	85%
Chocolate products	Chocolate	83%
Meat	Pâté	94%
Others	Soup	91%
	Rolled Oats	98%
	Spice Mix	102%

6. Surface validation

To test the ability of the kit to detect gluten in a stainless steel surface (collected with a cotton swab), the following procedure was followed: three "spiking" levels were chosen: high (65.5 ng gliadin/cm^2 or 131 ng gluten/cm^2), low (1.8 ng gliadin/cm^2 or 3.6 ng gluten/cm^2) and blank (untreated). Three different stainless-steel trays (one for each level) were prepared with 28 replicas each of 16 cm^2. Each 16 cm^2 square was thoroughly swabbed with a cotton swab. The swab was then cut and introduced in a vial. 1 mL of Dilution Solution was added, and the vial was vortexed for 1 min. The Dilution Solution was then assayed following the procedure. Number of assays was:

- 3 tray preparations for each level
- 2 assays per tray preparation (28 replicates of each level per tray)
- 3 different operators doing the collection

POD (probability of detection or probability of the method of giving a positive result) was calculated. POD is near 0 when the analyte is not present and should rise to 1 as the analyte concentration increases. We established a 95% confidence interval. We expected to obtain positive results in the low level of contamination in 75% to 95% of the cases and 100% positives in the high level of contamination. Qualitative results of these experiments are summarized in **Table 12**, including POD, LCL (lower control limit) and UCL (upper control limit).

The results show that there is between a 91% probability of detection of a low contaminated surface (1.8 ng gliadin/cm^2 or 3.6 ng gluten/cm^2) and 100% probability of detection of a highly contaminated surface (62.5 ng/cm^2 or 131 ng gluten/cm^2). The limit of detection (LoD) of the surface method was set to 1.8 ng gliadin/cm^2 (3.6 ng gluten/cm^2).

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Table 12. Qualitative results of surface analysis with GlutenTox ELISA Rapid G12					
Condition	**n total**	**n positives**	**POD**	**LCL**	**UCL**
Blank	32	0	0.00	0.00	0.11
Low (1.8 ng gliadin/cm^2 or 3.6 ng gluten/cm^2)	55	50	0.91	0.80	0.96
High (62.5 ng gliadin/cm^2 or 131 ng gluten/cm^2)	57	57	1.00	0.94	1.00

7. Analytical Sensitivity

To determine the analytical sensitivity, Dilution Solution was assayed 23 times in the inter-assay experiment. OD_{mean} and standard deviation was calculated. The corresponding concentration of the OD_{mean} + 5x standard deviation was defined as limit of detection. The corresponding concentration of the OD_{mean} + 10x standard deviation was defined as the analytical limit of quantification. Results are summarized in **Table 13.**

Table 13. Analytical sensitivity and limits of detection and quantification of GlutenTox ELISA Rapid G12		
	Blank replicates (n=23)	
0.050	**0.056**	
0.051	**0.059**	
0.055	**0.057**	
0.057	**0.062**	
0.063	**0.055**	
0.054	**0.058**	
0.058	**0.061**	
0.063	**0.052**	
0.050	**0.058**	
0.053	**0.052**	
0.054	**0.052**	
0.054		
Average	**SD**	**%CV**
0.056	**0.0039**	**7%**

		OD_{450nm}
Limit of Detection (LoD)	OD_{Blank} + 5x SD_{Blank}	**0.076**
Limit of Quantification (LoQ)	OD_{Blank} +10x SD_{Blank}	**0.096**

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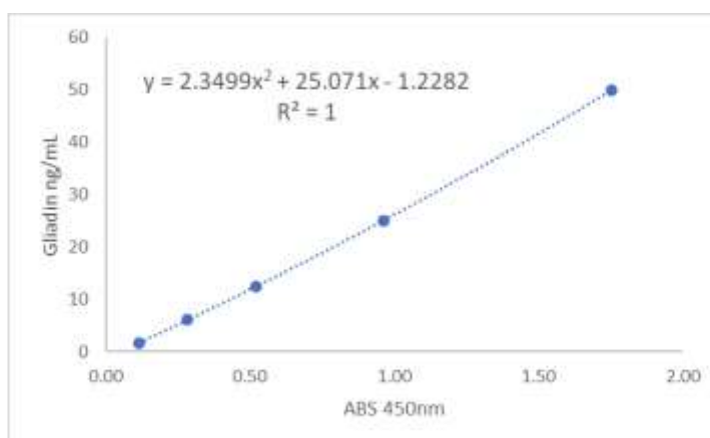

Figure 11. Graphical representation of the standard curve of the experiment to determine the analytical sensitivity of the assay.

The limit of detection (LOD) calculated for the kit is 0.27 ppm gluten, and it was rounded up to 0.3 ppm gluten.

The limit of quantification (LOQ) is defined by the lowest standard of the curve, and it is 0.6 ppm gluten. The analysis of the sensitivity confirms that it is over the analytical LOQ of the assay (0.48 ppm of gluten). This result confirms that the design of the standard curve is correct, and it is fitted for the purpose of the kit.

8. Cross-Reactivity

The foods shown in **Table 14** do not show any cross-reactivity (results <LoQ). The cross-reactivity analysis indicates that the positive results obtained in products that are safe for celiac patients are due to a gluten contamination and not to cross-reactivity.

Table 14. Foods with no cross-reactivity with GlutenTox ELISA Rapid G12

Almond flour	**Hazelnut flour**
Organic amaranth flour	Black lentil flour
Arrowroot powder	Lima bean organic flour
Gluten-free black bean flour	Powdered skimmed milk
Chestnut flour	Whole grain millet flour
Organic gluten-free coconut flour	Whole grain oat flour
Ground coffee	Potato starch
Corn starch	Whole grain quinoa flour
Freeze-dried bananas	Sesame seeds (hulled)
Dried egg powder	Wholegrain soy flour
Linseeds	Mixed Syrian spices
Gluten-free fava flour	Gluten-free white rice flour
Gluten-free green pea flour	Tapioca flour

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9. Robustness

Robustness study was performed to evaluate the ability of the method to remain unaffected by small variations in procedural parameters that might be expected to occur when the method is performed by an end user. Four parameters important to the end user were chosen to be varied **Table 15.**

Table 15. Ruggedness Parameters

Ruggedness Test Parameter	Low Value	Nominal Value (Package Insert Directions)	High Value
Extraction volume	4 mL	5 mL	6 mL
A1-HRP temperature before assay	-------------	2-8 ºC	15-25 ºC (2 hours)
A1-HRP incubation time	20 minutes	30 minutes	40 minutes
Substrate solution (TMB) incubation time	20 minutes	30 minutes	40 minutes

The effects of perturbations in *"Extraction volume"* were examined in rice pancake using the gliadin-containing reference material (PWG gliadin). Spikes were tested at four different spiked levels of gluten (0; 5; 20 and 40 ppm). The effects of perturbations in the rest of parameters *"Antibody conjugated to HRP (A1-HRP) temperature before starting the assay", "A1-HRP incubation time" and "Substrate solution (TMB) incubation time"* were examined in the standard curve.

When the *"Extraction volume" (4 or 6 mL, normal = 5 mL)* was tested **Table 16** the low value (4 mL) produced % recoveries (145-161%) above the normal value (5 mL) (108-118%) while the high value (6 mL) produced % recoveries (85-87%) below the normal value. These data indicated that the "Extraction volume" parameter cannot be diminished.

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Table 16. Ruggedness Parameter: Extraction volume

Matrix	Extraction volume (mL)	Spiking level (ppm gluten)	Result (ppm gluten, avg. of n=2)	SD	% CV	% Recovery
Rice pancake	4	0	<LQ	0.02	30%	<LQ
		5	7.85	0.00	0%	157%
		20	29.04	0.06	5%	145%
		40	64.56	0.08	4%	161%
	5	0	<LQ	0.02	42%	<LQ
		5	5.72	0.00	1%	114%
		20	21.52	0.05	6%	108%
		40	47.32	0.06	4%	118%
	6	0	<LQ	0.02	39%	<LQ
		5	4.32	0.00	1%	86%
		20	17.38	0.02	3%	87%
		40	34.11	0.04	3%	85%

When the *"Antibody conjugated to HRP (A1-HRP) remained two hours at room temperature before starting the assay"* **Table 17** the % recovery ranged from 91-106% (R^2 = 0.9999) and the coefficient of variation (%CV), compared to the normal value (2-8 ºC), ranged between 1% and 14% (except for the negative control) indicating that the assay was not significantly affected by this variation.

Table 17. Ruggedness Parameter: A1-HRP temperature before starting the assay

ng/mL gliadin	Std	2 hours room temperature A1-HRP	NORMAL VALUE	Avg.	SD	%CV
100	C+	2.32	2.63	2.47	0.21	9%
50	Std1	1.51	1.73	1.62	0.15	9%
25	Std 2	0.85	1.00	0.92	0.10	11%
12.5	Std 3	0.47	0.50	0.48	0.02	5%
6.25	Std 4	0.24	0.27	0.25	0.02	8%
1.56	Std 5	0.09	0.09	0.09	0.00	1%
9	IC	0.31	0.37	0.34	0.05	14%
	C-	0.01	0.03	0.02	0.01	67%

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When the *“Antibody conjugated to HRP (A1-HRP) incubation time” (20 or 40 minutes, normal=30 minutes)* Table 18 was examined the % CV, compared to the normal value, ranged from 8 to 31% when the incubation time was 20 minutes, and between 11 and 14 % when it was 40 minutes. These data indicated that the “A1-HRP incubation time” parameter cannot be shortened.

Table 18. Ruggedness Parameter: A1-HRP incubation time

ng/mL gliadin	Std	20 min incubation time A1-HRP	NORMAL VALUE	Avg.	SD	%CV
100	**C+**	**1.84**	**2.63**	2.23	0.56	25%
50	Std1	1.22	1.73	1.47	0.36	24%
25	Std 2	0.64	1.00	0.82	0.25	31%
12.5	Std 3	0.36	0.50	0.43	0.10	24%
6.25	Std 4	0.19	0.27	0.23	0.06	25%
1.56	Std 5	0.08	0.09	0.08	0.01	8%
9	IC	0.25	0.37	0.31	0.09	28%
	C-	0.04	0.03	0.03	0.00	9%
ng/mL gliadin	**Std**	**40 min incubation time A1-HRP**	**NORMAL VALUE**	**Avg.**	**SD**	**%CV**
100	**C+**	3.13	2.63	2.88	0.36	12%
50	Std1	2.06	1.73	1.89	0.23	12%
25	Std 2	1.16	1.00	1.08	0.11	11%
12.5	Std 3	0.61	0.50	0.55	0.07	14%
6.25	Std 4	0.31	0.27	0.29	0.03	11%
1.56	Std 5	0.10	0.09	0.10	0.01	10%
9	IC	0.44	0.37	0.40	0.04	11%
	C-	0.04	0.03	0.03	0.00	7%

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When the *"Substrate solution (TMB) incubation time" (20 or 40 minutes, normal=30 minutes)* **Table 19** was tested the % CV, compared to the normal value, ranged from 8 to 19% when the incubation time was 20 minutes, and between 1 and 5 % when it was 40 minutes showing, in this case, no statistical difference with the normal value. On the contrary, the assay was affected by a shorter incubation time (20 minutes).

Table 19. Ruggedness Parameter: Substrate solution (TMB) incubation time

ng/mL gliadin	Std	20 min incubation time TMB	NORMAL VALUE	Avg.	SD	%CV
100	**C+**	2.20	2.63	2.41	0.30	13%
50	Std1	1.38	1.73	1.55	0.24	16%
25	Std 2	0.76	1.00	0.88	0.17	19%
12.5	Std 3	0.40	0.50	0.45	0.07	17%
6.25	Std 4	0.21	0.27	0.24	0.04	16%
1.56	Std 5	0.08	0.09	0.08	0.01	8%
9	IC	0.30	0.37	0.34	0.05	16%
	C-	0.03	0.03	0.03	0.00	15%
ng/mL gliadin	**Std**	**40 min incubation time TMB**	**NORMAL VALUE**	**Avg.**	**SD**	**CV%**
100	**C+**	2.69	2.63	2.66	0.05	2%
50	Std1	1.80	1.73	1.76	0.05	3%
25	Std 2	0.96	1.00	0.98	0.02	2%
12.5	Std 3	0.52	0.50	0.51	0.02	3%
6.25	Std 4	0.28	0.27	0.27	0.01	3%
1.56	Std 5	0.10	0.09	0.09	0.00	5%
9	IC	0.38	0.37	0.38	0.00	1%
	C-	0.04	0.03	0.04	0.01	21%

10. Discussion

- The analytical validation shows a standard curve design that has an accuracy and precision values below 10% CV. The expanded uncertainty for the standard values is below 15%. The results show that the standard curve will provide

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reliable results for sample quantification in terms of linearity, repeatability and reproducibility.

- The kit does not present hook effect, so it assures that any positive sample no matter how high the contamination, will always be detected as positive.
- For the functionality assay, 12 different matrices were assessed. The results for precision and accuracy between the matrices assayed stay below 10% CV for accuracy and below 15% CV for precision.
- The average recovery of all matrices assayed ranges from 83% to 120%.
- The results of the Surface validation show that the kit can detect 1.8 ng gliadin/cm^2 (3.6 ng gluten/cm^2).
- None of the ingredients assayed for cross reactivity has yielded a positive result. Therefore, any positive result detected in those samples is linked to a gluten contamination and is not due to a non-specific signal.

- Robustness data indicated that minor variations in procedural parameters related to the reduction of the extraction volume or shortening the incubation times affected the GlutenTox ELISA Rapid G12 assay. However, the assay remained unaffected or was slightly affected by the lengthening of the incubation times or the increment of the extraction volume or when the antibody conjugated A1-HRP was left at room temperature for two hours before starting the analysis.

11. Conclusion

GlutenTox ELISA Rapid G12 is a new-generation kit for gluten determination in foods, drinks and surfaces. It uses the only two monoclonal antibodies (G12 and A1) 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.

The fact that two different antibodies are involved in the detection of gluten makes the kit highly specific for the selected target, the 33-mer. This specificity allowed a reduction in incubation time of 60 minutes compared to the previous version, making it one of the fastest kits in the market. This, in combination with zero restrictions in the number of wells to be used at the same time, thanks to the stability of the assay, grants a competitive advantage to GlutenTox ELISA Rapid G12 over other options.

It shows no hook (or prozone) effect, thus ensuring that there will be no false negatives due to highly contaminated samples. It has a very convenient surface protocol that rounds up the gluten-detection routine and ensures gluten-free working zones.

Critical reagents such as the GlutenTox A1-HRP antibody and the ready-to-use GlutenTox Standards show superb stability over time, conferring the kit with robust, reliable components that will withstand accidental temperature swings.

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