Validation Report
GlutenTox® ELISA Sandwich

KIT3011

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1. Scope

GlutenTox ELISA Sandwich is an immunosorbent assay for the detection and quantification of the toxic fraction of gluten that is harmful to celiac patients. The present report describes the validation process and its results.

GlutenTox ELISA Sandwich is based on the G12 and A1 antibodies, which specifically recognized the most immunotoxic fraction of gluten [1]. This is the 33-mer, a peptide within the α-gliadin molecule that triggers most of the immune response in celiac patients [2].

2. Analytical validation

a) Intra-Assay Variation

The intra-assay variation was determined by testing one curve in 11 replicates. See Table 1 for results.

Table 1. Analytical intra-assay variation of GlutenTox ELISA Sandwich														
	OD at 450nm generated by the standards													
ng/mL gliadin	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 5**	**Rep 6**	**Rep 7**	**Rep 8**	**Rep 9**	**Rep 10**	**Rep 11**	**Avg.**	**SD**	**CV%**
100	2.63	2.63	2.66	2.80	2.59	2.62	2.46	2.69	2.77	2.70	2.83	**2.67**	0.12	4
50	1.84	1.93	1.86	1.79	1.82	1.68	1.54	1.71	1.75	1.79	1.98	**1.79**	0.11	6
25	1.18	1.22	1.29	1.32	1.09	1.16	1.19	1.12	1.09	1.20	1.27	**1.19**	0.09	8
12.5	0.79	0.76	0.83	0.81	0.79	0.81	0.79	0.82	0.76	0.95	0.86	**0.81**	0.02	3
6.25	0.46	0.48	0.48	0.55	0.51	0.48	0.49	0.52	0.49	0.50	0.49	**0.50**	0.03	5
3.12	0.27	0.27	0.28	0.31	0.31	0.30	0.30	0.28	0.29	0.28	0.27	**0.29**	0.01	4
1.56	0.19	0.19	0.20	0.21	0.21	0.20	0.21	0.20	0.21	0.21	0.21	**0.20**	0.00	2
Blank	0.08	0.08	0.08	0.09	0.08	0.08	0.09	0.08	0.09	0.09	0.09	**0.08**	0.00	6
Avg. CV%														5

The coefficient of variation (CV) ranges from 2% to 8% depending on the concentration of the Standard. The average CV is 5%, well below the 20% of acceptance criteria.

b) Inter-Assay Variation

The inter-assay variation was determined by testing six different test runs of the same kit lot. See Table 2 for results.

Table 2. Analytical inter-assay variation of GlutenTox ELISA Sandwich															
	Assay 1		Assay 2		Assay 3		Assay 4		Assay 5		Assay 6				
ng/mL gliadin	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Avg.	SD	CV%
100	2.30	2.37	2.39		2.85	2.85	2.47	2.61	2.49	2.54	2.63	2.63	**2.56**	0.18	7%
50	1.76	1.79	1.90	1.88	2.19	2.15	1.97	2.02	1.90	1.85	1.84	1.93	**1.93**	0.13	7%
25	1.24	1.30	1.38	1.55	1.61	1.57	1.41	1.52	1.33	1.42	1.18	1.22	**1.39**	0.15	10%
12.5	0.87	1.04	0.93	1.03	1.07	1.09	0.91	0.98	0.88	0.93	0.79	0.76	**0.94**	0.11	11%
6.25	0.51	0.57	0.60	0.56	0.64	0.64	0.59	0.59	0.38	0.36	0.46	0.48	**0.53**	0.09	17%
3.12	0.33	0.32	0.33	0.35	0.38	0.39	0.35	0.37	0.28	0.29	0.27	0.27	**0.33**	0.04	13%
1.56	0.23	0.24	0.23	0.24	0.24	0.24	0.24	0.25	0.22	0.23	0.19	0.19	**0.23**	0.02	9%
Blank	0.08	0.09	0.08	0.08	0.09	0.09	0.08	0.09	0.10	0.10	0.08	0.08	**0.09**	0.01	8%
Avg. CV%															10%

The coefficient of variation ranges from 7% to 17% depending on the concentration of the Standard. The average CV is 10%, below the 20% of acceptance criteria

c) Inter-lot Variation

Inter-lot variation was determined by testing three different batches of standards. See Table 3 for results.

Table 3. Inter-lot variation of 3 batches of GlutenTox Standards						
ng/mL gliadin	Average OD batch 191050	Average OD batch 191250	Average OD batch 180151	Average	SD	CV%
100	2.24	2.25	2.59	2.36	0.20	8%
50	1.61	1.68	1.88	1.72	0.14	8%
25	1.18	1.21	1.26	1.22	0.04	4%
12.5	0.77	0.83	0.88	0.83	0.05	6%
6.25	0.62	0.61	0.54	0.59	0.04	8%
3.12	0.34	0.34	0.32	0.33	0.01	4%
1.56	0.21	0.21	0.22	0.21	0.00	2%
Blank	0.09	0.09	0.10	0.09	0.01	10%
Avg. CV%						**5%**

The coefficient of variation ranges from 2% to 8% depending on the concentration of the Standard. The average CV is 5%, well below the 20% acceptance criteria.

3. Recovery

For recovery experiments several blank matrices (cereals, fried snacks, flours, chocolate and spices) were spiked with gliadin to obtain various final concentrations (3 ppm, 20 ppm and 100 ppm gluten). Spiked samples were extracted following manual instructions and assayed. Intra-assay, inter-assay and recovery experiments were carried out. Results are summarized in Tables 4-6.

Table 4. Intra-assay variation of 5 matrices with GlutenTox ELISA Sandwich

Matrix	Spiking level (mg/kg gluten)	Average recovery (n=9)	CV%
Cereals	3	2.3	4.5
Cereals	20	20.3	6.1
Cereals	100	79.8	7.8
Flours	3	2.2	2.3
Flours	20	21.9	7.4
Flours	100	93.2	13.9
Fried snacks	3	3.0	9.6
Fried snacks	20	21.5	6.7
Fried snacks	100	84.6	8.9
Chocolate	3	2.3	4.6
Chocolate	20	15.8	2.9
Chocolate	100	106.0	8.0
Spices	3	2.2	5.1
Spices	20	19.0	7.6
Spices	100	85.2	11.1

Table 5. Inter-assay variation of 5 matrices with GlutenTox ELISA Sandwich			
Matrix	**Spiking level (mg/kg gluten)**	**Average recovery (n=15)**	**CV%**
Cereals	3	2.4	14.5
Cereals	20	19.0	12.6
Cereals	100	82.0	8.7
Flours	3	2.5	13.2
Flours	20	23.4	10.2
Flours	100	86.8	7.9
Fried snacks	3	2.8	9.9
Fried snacks	20	20.6	13.5
Fried snacks	100	84.5	12.2
Chocolate	3	2.4	6.8
Chocolate	20	16.0	4.6
Chocolate	100	97.7	12.5
Spices	3	2.3	9.6
Spices	20	17.7	11.4
Spices	100	86.2	12.0

Table 6. Percentage of recovery of 5 matrices and 3 spiking levels with GlutenTox ELISA Sandwich			
Matrix	**Spiking level (mg/kg gluten)**	**Concentration**	**Recovery%**
Cereals	3	2.3	75.5
Cereals	20	20.3	101.4
Cereals	100	79.8	79.8
Flours	3	2.2	81.4
Flours	20	21.9	109.4
Flours	100	93.2	93.2
Fried snacks	3	3.0	99.1
Fried snacks	20	21.5	107.4
Fried snacks	100	84.6	84.6
Chocolate	3	2.3	76.3
Chocolate	20	15.8	79.2
Chocolate	100	106.0	106.0
Spices	3	2.2	74.2
Spices	20	19.8	88.4
Spices	100	85.2	82.0

Confirmation experiments were done with the current set of standards. 2 sets of corn flour were spiked at 10, 20 and 30 ppm of gluten and analyzed with 18 different batches of antibody G12-HRP.

Table 7. Percentage of recovery experiments with corn flour at 3 spiking levels with GlutenTox ELISA Sandwich

Spiking level (mg/kg gluten)	Average quantification	SD	CV %	% Recovery
10	9.37	1.18	13%	94%
20	19.90	1.94	10%	100%
30	29.67	3.74	13%	99%
10	10.18	1.76	17%	102%
20	23.62	2.81	12%	118%
30	33.83	5.44	16%	113%
Avg. CV%			**13%**	
Avg. Recovery				**104%**

4. Analytical Sensitivity

For determination of the analytical sensitivity sample diluent was assayed in 16-fold replicates. After identification of possible outliers, the OD_{mean} and standard deviation was calculated. The corresponding concentration of the OD_{mean} + 3x standard deviation was defined as limit of detection. The corresponding concentration of the OD_{mean} + 10x standard deviation was defined as limit of quantification.

Table 8. Analytical sensitivity and limits of detection and quantification of GlutenTox ELISA Sandwich

Blank replicates		
0.084	0.086	
0.076	0.088	
0.082	0.091	
0.085	0.083	
0.079	0.089	
0.082	0.087	
0.094	0.084	
0.083	0.085	
Average	**SD**	**CV %**
0.085	**0.004**	**5%**

		OD 450nm	Gliadin (ng/mL)
Limit of Detection (LoD)	OD_{Blank} + 3x SD_{Blank}	**0.098**	**0.30**
Limit of Quantification (LoQ)	OD_{Blank} +10x SD_{Blank}	**0.129**	**0.73**

5. Cross Reactivity

For the following foods, no cross-reactivity (results <LoQ) could be detected:

Table 9. Foods with no cross-reactivity with GlutenTox ELISA Sandwich	
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

6. External studies

In a study performed in RIKILT-Wageningen University and Research in 2015, the authors evaluated and compared a set of 14 ELISA kits for gluten detection. GlutenTox ELISA Sandwich results are summarized in Table 10.

Table 10. Recovery of a 10mg/kg spiking with PWG Gliadin in different matrices with GlutenTox ELISA Sandwich

Matrix	Spiking level (mg/kg)	Concentration
Baby Food	0	<0.3
Baby Food	10	10.1±0.1
Spice Mix	0	<0.3
Spice Mix	10	10.2±1.0
Soy Sauce	0	<0.3
Soy Sauce	10	15.5±0.1

7. 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] BRUINS SLOT, I et al., “Evaluating the performance of Gluten ELISA Test Kits: the numbers do not tell the tale”. Cereal Chemistry, (2015). https://doi.org/10.1094/CCHEM-07-14-0166-R