INgezim GLUTEN

R.30.GLU.K.2

Double Antibody Sandwich (DAS) ELISA kit for the detection of Gluten residues.

Last revision date: Dec- 2018

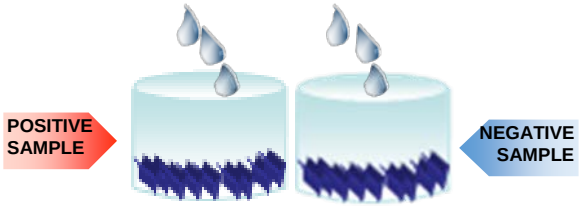
1 PRODUCT APPLICATION

The product INgezim GLUTEN has been designed for quantitative analysis of Gluten proteins in food and environmental samples.

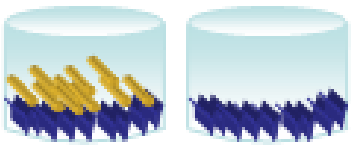
2 TECHNICAL BASIS OF THE PRODUCT

The product is based on the Double Antibody Sandwich (DAS) ELISA technique, which is summarized in the following scheme.

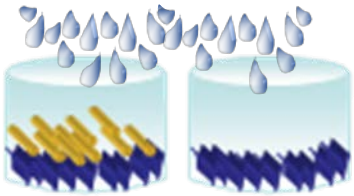
1. Plates are supplied coated with **R5** monoclonal antibodies specific for Prolamins. On these wells, samples are added and incubated



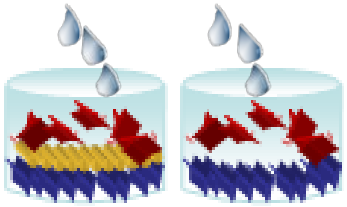
2. If samples contain the antigen, it will bind the antibodies specific for Prolamins, which are coating the plate.



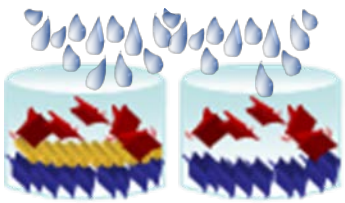
3. At this point a washing step is needed to remove all non-specifically fixed materials



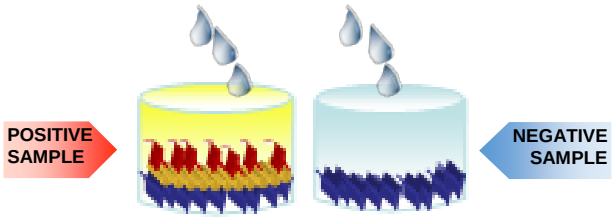
4. If we add the R5 antibody specific for Gluten, it will bind to the antigen of the sample. For future detection of this antigen, this antibody is labelled with Peroxidase.



5. Again a washing step is required to remove all non specifically fixed conjugate



6. If a substrate for peroxidase is added, a colorimetric reaction will appear.



3 KEY REAGENTS USED

The optimum performance of the test is partially due to the key reagents used in its formulation, which, are:

- Capture Monoclonal Antibody: R5 monoclonal antibody specific for prolamins of wheat, rye and barley (no oats).
- Conjugate: R5 monoclonal antibody specific for prolamins of wheat, rye and barley (no oats) HRPO (Horseradish peroxidase) conjugated.

4 REFERENCE MATERIAL

EUROPEAN GLIADIN FROM THE PWG	
Product Description	Reference gliadine (lyophilized)
Supplier	Prolamin Working Group
Available Presentations	Vial of 100 mg
Storage	4ºC

5 VALIDATION PROTOCOL

The production of every new lot implies the following internal validation steps:

a. Preparation and validation of the standard curve. The OD of every standard should meet pre-fixed values.
b. Validation of internal and external reference samples (FAPAS). Following the kit insert, every sample should meet a pre-fixed value according with the standard points.
c. Preparation and validation of the Conjugate, according with the correspondent standard points and Negative Control
d. The absorbance value of the Positive Control should be higher than the absorbance of the 25ng/ml point
e. The absorbance value of the Negative Control should be lower that the absorbance of the 1,56 ng/ml point
f. Variability of the standard points and reference samples
g. Intraplate variability
h. Interplate variability

6 SENSITIVITY

6.1 LIMIT OF DETECTION AND QUANTIFICATION USING THE EUROPEAN STANDARD

In order to determine the sensitivity of the assay, the European standard was used. Five different dilutions of it were made. The results obtained showed that the assay was able to detect 1,56ng/ml of this standard. This value corresponds to an amount of **3ppm of gluten** in foods according to the next formula and supposing a sample dilution of 1/25.

ppm = (C x D x 2 x 40) /1000

Where:

C: concentration of the sample calculated from the calibration curve in ng/ml.

D: Dilution factor of the sample (25, 50, 100, etc.)

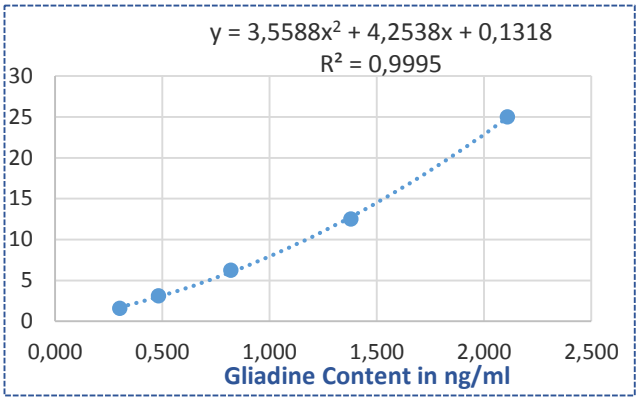
2: Applied factor to express the results in gluten concentration

40: Dilution factor used during the sample preparation (0,25g in 10 ml of extraction buffer).

1000: conversion from ng/ml to ppm

TABLE I

STANDARDS (ng/mL)	OD (450)
25	2,109
12,5	1,378
6,25	0,819
3,12	0,482
1,56	0,301



6.2 LIMIT OF QUANTIFICATION IN FOOD MATRIX

Five Gluten Free samples were fortified at the lowest level of quantification of the assay (**3 ppm of Gluten**). All samples were assayed 3 times, obtaining the results showed in the Table below (Table II)

TABLE II (Recovery rates at LOQ level)

Matrix	Assay 1 (ppm)	Assay 2 (ppm)	Assay 3 (ppm)	Average	CV (%)
York ham	3.2	3.2	2.4	2.9	15
Baby food (with 6 fruits)	2.6	4.86	3.04	3.5	34
Soy milk	4.9	3.02	3.52	3.8	25
Gluten free cookies	4.6	4.3	4.2	4.4	5
Chocolate with milk	2.9	2.3	2.01	2.4	19

The presence of gluten at the limit of quantification (3 ppm) was reliably detected in all cases; as the absorbance values of the fortified samples were higher than the negative control, blank for reagents, and the correspondent non fortified samples.

7 SPECIFICITY AND SELECTIVITY

The specificity of the R5 Monoclonal Antibody used in the assay was determined by the Unit of Gluten of the National Center of Biotechnology (CNB); by using immunoblotting technique. This R5 MAb specifically detects the prolamins of gluten present in WHEAT, RYE and BARLEY (gliadins, secalin and hordein respectively).

For checking the specificity and cross-reactivity of the assay, 50 samples (free of gluten) were extracted and analysed, following the instructions of the INGEZIM GLUTEN kit (30.GLU.K2). The results are summarized in the table below (Table III)

TABLE III (Specificity and Cross-Reactivity)

	MATRIX	Result
1	Sweetener (powder)	Negative (< 3 ppm)
2	Sweetener (tablets)	Negative (< 3 ppm)
3	Brewer's yeast	Negative (< 3 ppm)
4	Chips (maize)	Negative (< 3 ppm)
5	Snacks	Negative (< 3 ppm)
6	Snacks with cheese flavor	Negative (< 3 ppm)
7	Black chocolate (74%)	Negative (< 3 ppm)
8	White chocolate	Negative (< 3 ppm)
9	Melted chocolate	Negative (< 3 ppm)
10	Hot chocolate	Negative (< 3 ppm)
11	Mint tea	Negative (< 3 ppm)
12	Gluten free baby food (Milupa)	Negative (< 3 ppm)
13	Gluten free baby food (Nestlé)	Negative (< 3 ppm)
14	Yeast for cooking	Negative (< 3 ppm)
15	Gluten free rustic bread	Negative (< 3 ppm)
16	Gluten free baby food (dextrinated cereals)	Negative (< 3 ppm)
17	Gluten free baby food (Nutricia)	Negative (< 3 ppm)
18	Gluten free baby food (Nutriben)	Negative (< 3 ppm)
19	Custard	Negative (< 3 ppm)
20	Gelatin	Negative (< 3 ppm)
21	Mize flour	Negative (< 3 ppm)
22	Fish soup	Negative (< 3 ppm)
23	Rice crackers with chocolate	Negative (< 3 ppm)
24	Cereals mixture (rice, maize and dry fruits)	Negative (< 3 ppm)
25	Cornstarch	Negative (< 3 ppm)
26	Rice flour	Negative (< 3 ppm)
27	Rice semolina	Negative (< 3 ppm)
28	Custard with canola	Negative (< 3 ppm)
29	White chocolate	Negative (< 3 ppm)
30	Gluten free pre-cooked bread	Negative (< 3 ppm)
31	Cereals with coconut	Negative (< 3 ppm)
32	Milk chocolate	Negative (< 3 ppm)
33	Rice crackers with soy	Negative (< 3 ppm)
34	Maize crackers	Negative (< 3 ppm)
35	White chocolate with nuts	Negative (< 3 ppm)

36	Milk chocolate with almonds	Negative (< 3 ppm)
37	Black chocolate (60%)	Negative (< 3 ppm)
38	Quick yeast for cooking	Negative (< 3 ppm)
39	Cornflour	Negative (< 3 ppm)
40	Chicken bouillon (tablet)	Negative (< 3 ppm)
41	Meat bouillon (tablet)	Negative (< 3 ppm)
42	Fish bouillon (tablet)	Negative (< 3 ppm)
43	Sweet pepper	Negative (< 3 ppm)
44	Rice crackers with caramel	Negative (< 3 ppm)
45	Gluten free flour	Negative (< 3 ppm)
46	Cookies with chocolate (gluten free)	Negative (< 3 ppm)
47	Corn with honey	Negative (< 3 ppm)
48	Baby food with gluten free cereals	Negative (< 3 ppm)
49	Tea with lemon	Negative (< 3 ppm)
50	Crème caramel	Negative (< 3 ppm)

8 RECOVERY DATA

8.1 RECOVERY USING REFERENCE SAMPLES

Some reference samples were analyzed by using the INGEZIM GLUTEN assay. These samples includes fortified samples (spikes), FAPAS samples and reference samples from other laboratories. Results are showed in the Table below (Table IV):

TABLE IV (RECOVERY OF REFERENCE SAMPLES)

Sample	Theoretical Gluten content (ppm)	INGEZIM GLUTEN result (ppm)	Recovery (%)
Spike 0 ppm	0	< 1.56	-
Spike 20 ppm	20	24	**120**
Spike 50 ppm	50	58	**116**
Spike 100 ppm	100	114	**114**
Spike 200 ppm	200	196	**98**
Fapas Cake Mix	20.5	22	**107**
Fapas 20 ppm	20	22	**110**
M 104 65 ppm	65	54	**83**
10 ppm A+	10	12	**120**
100 ppm A+	100	105	**105**
M 83 21 ppm	21	25	**119**
M 93 25 ppm	25	28	**112**
Fapas 2795A	70	71	**101**

8.2 RECOVERY AT 20 ppm

A range of matrixes was fortified with 10 ppm of gliadin (20 ppm of Gluten). Results by using INGEZIM GLUTEN kit are showed in the table below (Table V).

TABLE V (Recovery data at 20ppm spiking level)

SAMPLES	ppm
Soy drink with cinnamon and lemon	26
Vegetables juice	22
Pineapple drink with soy	17
Orange and carrot juice	23
Tiger nut milk drink (horchata)	24
Coconut milk	29
Bolognese sauce	19
Almond drink	23
Vinaigrette	19
Ketchup	21
Barbecue sauce	24
Fried tomato	26
Mustard sauce	30
Orange juice	23
Vegetable soap	22
Rice drink	28
Soy drink with papaya and mango	23
Soy drink with chocolate	16
Balsamic vinaigrette with honey	18

9 VARIABILITY

9.1 STANDARD CURVE VARIABILITY

The standards of three different kits were assayed by duplicate several times and at different days, in order to assess the absorbance variability. Results are showed in the table below:

TABLE (Standards curve absorbance at 450 nm)

	Positive Control	25 ng/mL	12,5 ng/mL	6,25 ng/mL	3,12 ng/mL	1,56 ng/mL	Negative Control	Blank of reagents
Kit 1 (Assay 1)	3.096	2.260	1.637	0.984	0.677	0.443	0.157	0.157
Kit 1 (Assay 2)	3.025	2.148	1.500	0.961	0.642	0.431	0.146	NA
Kit 1 (Assay 3)	3.212	2.575	1.824	1.216	0.744	0.514	0.149	0.138
Kit 2 (Assay 1)	3.325	2.421	1.936	1.246	0.755	0.514	0.194	0.166
Kit 2 (Assay 2)	2.735	2.184	1.528	0.974	0.678	0.480	0.174	0.167
Kit 3 (Assay 1)	3.373	2.546	1.833	1.138	0.729	0.488	0.262	0.137
Kit 3 (Assay 2)	3.104	2.292	1.862	1.106	0.681	0.396	0.187	0.148
Kit 3 (Assay 3)	NA	2.181	1.599	1.024	0.673	0.407	0.251	0.141
Average	3.124	2.326	1.715	1.081	0.697	0.459	0.190	0.150
CV %	**6.8**	**7.2**	**9.8**	**10.4**	**5.7**	**10.1**	**23.4**	**9.2**

The CV % for the duplicates was < 10% in all the cases. In addition, there was no significant differences comparing the absorbance values of the standards between different kits and different assays.

9.2 INTRAPLATE VARIABILITY

The same point was analysed in all the wells of the same ELISA plate.
Absorbance values and variability data are showed below:

Intraplate variability											
1,15	1,111	1,093	1,136	1,042	1,144	1,136	1,071	1,204	1,11	1,186	1,103
1,099	1,051	1,046	0,933	1,046	1,052	1,04	1,12	1,088	1,155	1,176	1,103
1,111	1,05	1,01	0,989	1,005	1,01	1,011	1,052	1,065	1,142	1,161	1,13
1,139	1,183	0,985	0,975	1	0,991	0,996	1,035	1,08	1,135	1,146	1,145
1,132	1,062	1,017	1,028	1,032	1,187	1,037	1,061	1,109	1,143	1,163	1,152
1,126	1,073	1,022	1,042	1,017	1,02	1,034	0,948	1,079	1,112	1,162	1,142
1,195	1,108	1,075	1,1	1,121	1,113	1,111	0,983	1,146	1,182	1,191	1,162
1,02	1,15	1,148	1,169	1,153	1,202	1,187	1,117	1,123	1,209	1,182	1,112

Average	σ	CV
1,094	0,066	**6,04**

9.3 INTERPLATE VARIABILITY

The same point was analysed in 12 strips wells, every strip from a different plate.
Absorbance values and variability data are showed below:

Interplate variability											
Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Plate 7	Plate 8	Plate 9	Plate10	Plate11	Plate12
1,093	1,046	0,948	1,041	0,952	1,051	1,062	1,098	1,13	1,117	1,135	1,076
1,024	1,001	0,921	0,954	0,996	0,974	0,984	0,99	1,021	1,032	0,961	1,119
1,036	0,934	0,911	0,901	0,939	0,943	0,959	0,947	1,006	0,996	1,038	1,119
0,913	0,967	0,908	0,905	0,922	0,915	0,928	0,932	1,012	1,003	1,059	1,12
1,058	0,961	0,915	0,923	0,952	0,965	0,971	0,987	1,011	1,044	1,106	1,079
1,024	0,958	0,914	0,958	0,933	0,917	0,948	0,956	0,965	1,016	1,118	1,153
1,101	1,042	1,017	0,939	1,027	1,017	0,934	0,971	1,055	1,128	0,982	1,124
0,962	1,1	1,06	1,084	1,061	1,053	1,102	1,115	1,1	1,102	1,107	1,143

Average	σ	CV
1,028	0,069	**6,73**

9.4 INTRA EXTRACTION VARIABILITY

Sample A and Sample B were extracted, one time each. Both extractions were tested 5 times each, obtaining the results showed below

	Sample A	
	OD	Gluten (ppm)
Exp #		
Nº 1	1,01	10,0
Nº2	0,98	13,3
Nº3	1,11	12,9
Nº4	1,14	11,3
Nº5	1,00	10,4
Mean	1,05	11,60
Standard Dev.	0,07	1,31
VC*	6,79	11,37

	SampleB	
	OD	Gluten (ppm)
Exp #		
Nº 1	0,895	1,9
Nº2	0,785	2,1
Nº3	0,989	2,6
Nº4	0,979	2,1
Nº5	0,851	1,9
Mean	0,90	2,10
Standard Dev.	0,09	0,25
VC*	9,59	12,08

9.5 INTER EXTRACTION VARIABILITY

Samples A and B were extracted at 5 different days. Obtained results are showed below:

	SampleA	
	OD	Gluten (ppm)
Exp #		
Nº 1	1,00	9,7
Nº2	0,97	13,1
Nº3	0,98	10,0
Nº4	1,03	9,2
Nº5	0,89	8,0
Mean	0,97	10,00
Standard Dev.	0,05	1,69
VC*	5,31	16,90

	Sample B	
	OD	Gluten (ppm)
Exp #		
Nº 1	0,908	1,9
Nº2	0,86	2,6
Nº3	0,918	2,2
Nº4	0,95	1,9
Nº5	0,82	1,7
Mean	0,89	2,06
Standard Dev.	0,05	0,31
VC*	5,75	15,20

9.6 INTERBATCH VARIABILITY USING REFERENCE SAMPLES

Reference samples with a known gluten content, were analysed by using 3 different lots of INGEZIM GLUTEN kits.
Average results, standard deviation and CV are showed in the table below:

Reference Sample	Batch A	Batch B	Batch C	Average	σ	CV
Sample 1 (10 ppm)	11	9	9	9,6	1,1	11,9
Sample 2 (20 ppm)	21	19	23	21	2	9,5
Sample 3 (100 ppm)	99	93	100	97	3,7	3,8

10 PRODUCT LIMITATION

The assay is not designed for being used with samples where the GLUTEN proteins have been hydrolysed by any kind of sample treatment.

The most common hydrolysed samples are beer, whiskey, some syrups and some baby food.

For this kind of samples, INGEZIM HYDROLYSED GLUTEN (30.GLH.K2) assay should be used.

11 FAPAS PROFICIENCY TESTING RESULTS

The performance of the INgezim Gluten assay (30.GLU.K2) has been tested by participating in some proficiency testing (FAPAS); and obtaining equivalent results compared with other kits from the market (including our INgezim Gluten Quick kit 30.GL2.K2)

The assigned value (x_a) was determined for each analyte where sufficient data allowed and, in conjunction with the standard deviation for proficiency (σ_p) was used to calculate a z-score for each result.

Results for the proficiency tests are summarized in the tables below:

Proficiency test 27227	Assigned value, x_a mg/mL	Number of scores, $\|z\|\leq 2$	Total number or scores	% $\|z\|\leq 2$
Ingenasa INgezim Gluten (30.GLU.K2)	52.0	10	12	83
Ingenasa INgezim Gluten Quick (30.GL2.K2)	56.0	7	7	100
Neogen Veratox for Gliadin R5 (8510)	37.3	14	16	88
R-Biopharm Ridascreen Gliadin (R7001)	50.0	90	97	93
R-Biopharm Ridascreen Fast Gliadin (R7002)	46.2	6	6	100
Romer Labs AgraQuant ELISA Gluten G12 (COKAL02000)	50.7	3	3	100

Proficiency test 27228	Assigned value, x_a mg/mL	Number of scores, $\|z\|\leq 2$	Total number or scores	% $\|z\|\leq 2$
Ingenasa INgezim Gluten (30.GLU.K2)	54.0	9	9	100
Ingenasa INgezim Gluten Quick (30.GL2.K2)	56.0	6	6	100
Neogen Veratox for Gliadin R5 (8510)	30.0	9	9	100
R-Biopharm Ridascreen Gliadin (R7001)	45.0	52	58	90