FOOD COMPOSITION AND ADDITIVES

Validation Study of the Veratox R5 Rapid ELISA for Detection of Gliadin

***Performance Tested Method*SM 061201**

Abstract

Neogen Corp. developed the Veratox R5 Gliadin test kit for the detection of gliadin based on the monoclonal antibody R5 developed by Enrique Mendez (1). The purpose of this study was to validate the method under the requirements of the AOAC Research Institute *Performance Tested Method*SM program. There are two AOAC *Official Methods*SM for gluten detection in foods, **991.19** and **2012.01** (2), both of which are ELISAs. With the R5 Mendez method listed in the CODEX Alimentarius as a type 1 method for the detection of gluten in foods, the need to have additional rapid test kits validated by the AOAC Research Institute exists.

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The method was independently tested, evaluated, and certified by the AOAC Research Institute as a *Performance Tested Method*SM. *See* http://www.aoac.org/testkits/steps.html for information on certification.

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Scope of Method

The Veratox R5 Gliadin test kit is designed to detect gliadin in a variety of matrixes represented by those listed below at a range of 2.5–40.0 ppm. This range is suitable for proposed gluten-free monitoring in the United States and is compliant with current Codex Alimentarius definitions (3).

Target analyte.—Prolamins from wheat (gliadin), barley (hordein), and rye (secalin).

Matrixes.—Bread, rice flour, cooked hamburger, and breakfast cereal.

Summary of validated performance claims based on independent validation study:

Precision.—Mean RSD: 22.25% for breakfast cereal, 17.96% for cooked hamburger, 13.92% for bread, and 18.84% for rice flour across all levels tested.

Recovery.—Mean recovery: 102.60% for breakfast cereal, 105.79% for cooked hamburger, 110.65% for bread, and 105.87% for rice flour across all levels tested.

Cross reactivity with closely related compounds.—None.

LOD.—2.16 ppm breakfast cereal, 1.21 ppm cooked hamburger, 0.63 ppm bread, and 1.23 ppm rice flour.

Range of quantitation.—This assay has a range of quantitation between 2.5 and 40 ppm reported on a gliadin scale without additional dilution. The range can be extended through further dilution.

Definitions

Precision.—RSD of multiple assays of the same sample where RSD = (SD/Mean × 100%).

$$\text{Recovery \%} = [\text{mean}_{cand}/\text{true}] \times 100$$
(cand = candidate method).

LOD.—Defined as the mean value of 10 negative samples + 3.3 SDs.

Range of quantitation.—Range at which the results obtained can be accurate.

Introduction

Gliadin is an alcohol-soluble protein found in wheat that belongs to a group of proteins called prolamins. Other prolamins include secalin, found in rye, and hordein, found in barley. Gluten consists of two groups of proteins (prolamins and glutelins) found in differing amounts in wheat, barley, rye, and oats.

Gliadin and other prolamins have been identified as major causal agents in a number of disorders, including wheat allergy (Celiac disease) and gluten intolerance. Wheat allergy is a specific immune response to a number of wheat proteins,

Table 1. Internal spike and recovery results[a]

	No spike	5 ppm	10 ppm	25 ppm
		Rice		
Raw data	0.00	6.80	12.00	28.40
	0.10	6.80	11.40	26.70
	0.00	6.70	11.20	25.60
Average	0.00	6.77	11.53	26.90
SD		0.06	0.42	1.41
RSD, %		1.50	3.48	5.20
Recovery, %	NA[b]	136.00	115.00	108.00
		Oats		
Raw data	0.00	4.80	9.40	20.70
	0.20	4.80	8.64	18.72
	0.00	4.80	9.40	17.30
Average	0.00	4.80	9.15	18.91
SD		0.00	0.44	1.71
RSD, %		0.00	4.80	9.03
Recovery, %	NA	96.00	91.00	76.00
		Sorghum flour		
Raw data	1.90	6.30	12.40	34.20
	2.10	6.20	12.70	29.10
	0.00	6.50	12.80	22.10
Average	1.33	6.33	12.63	28.47
SD		0.15	0.21	6.07
RSD, %		2.41	1.65	21.34
Recovery, %	NA	126.00	126.00	114.00
		Corn		
Raw data	0.30	4.00	8.00	17.90
	0.20	4.10	7.90	16.00
	0.40	4.60	8.90	18.50
Average	0.70	4.23	8.27	17.47
SD		0.32	0.55	1.31
RSD, %		7.59	6.66	7.47
Recovery, %	NA	84.00	83.00	70.00
		Buckwheat		
Raw data	0.00	4.80	10.70	29.30
	0.00	5.00	10.60	29.60
	0.00	5.30	10.20	32.50
Average	0.00	5.03	10.50	30.47
SD		0.25	0.26	1.77
RSD, %		5.00	2.52	5.80
Recovery, %	NA	100.00	105.00	122.00
		Sausage		
Raw data	1.00	5.90	13.60	37.00
	0.20	5.90	12.00	33.00
	0.10	6.00	12.00	43.10
Average	0.40	5.93	12.53	37.70
SD		0.06	0.92	5.09
RSD, %		0.97	7.37	13.49
Recovery, %	NA	118.00	125.00	151.00
		Cracker		
Raw data	0.40	5.50	12.80	35.70
	0.80	6.21	12.40	37.20
	0.50	5.40	12.60	33.00
Average	0.40	5.70	12.60	35.30
SD		0.44	0.20	2.13
RSD, %		7.74	1.59	6.03
Recovery, %	NA	114.00	126.00	141.00
		Breakfast cereal		
Raw data	0.00	6.70	12.70	18.20
	0.00	6.80	11.90	17.90
	0.30	7.00	11.90	17.80
Average	0.40	6.83	12.17	17.97
SD		0.15	0.46	0.21
RSD, %		2.24	3.80	1.16
Recovery, %	NA	136.00	122.00	72.00
Overall recovery = 110.70%				
Overall RSD = 5.20%				

[a] All results in ppm.

[b] NA = Not applicable.

including gliadin, albumin, globulin, and glutenin. Celiac disease is a chronic reaction to gluten proteins that results in inflammation and the poor absorption of nutrients in the small intestine.

Those who must avoid gluten rely upon the correct labeling of food to make appropriate, safe food choices. Testing for the presence of gluten components ensures food manufacturers that an unlabeled—and potentially dangerous—ingredient did not make its way into a food product.

Principle

Veratox R5 Gliadin test kit is a sandwich ELISA. Gliadin is extracted from samples with an 80% ethanol solution by shaking in a shaker or rotator. Extract is diluted in phosphate saline buffer (PBS), and diluted samples are added to R5 antibody-coated wells (capture antibody), where gliadin will bind to the antibody during an incubation period. Any unbound gliadin and other components are washed away, and a second antibody, R5 enzyme-labeled (detector antibody), is added. The detector antibody binds to the gliadin during another incubation period. Unbound enzyme-labeled antibody is washed away, and a one-step substrate is added. Color develops as a result of the presence of bound-labeled antibody. A stopping reagent is added, and the color of the solution is observed. Blue color indicates samples containing high levels of gliadin, while purple or red samples contain little or no gliadin. The optical densities

Table 2. Comparison to existing methods

	Mean values (ppm)	
Samples	Neogen	*Official Method* **2012.01**
Soy flour	14.20	16.20
Rice cereal	49.50	62.70
Rice crisp	73.70	71.80
Granola bars	33.50	43.40
Rice cereal	57.80	64.00
Rice mix	63.10	61.60
Soy crisp	30.00	30.90
Ginger cake mix	46.60	48.60
Oat cereal	29.60	29.10
Chocolate wafers	40.30	40.10
Rice cocoa cereal	38.50	42.20
Multigrain cereal	85.20	84.00
Corn flakes cereal	9.00	10.60
Chocolate oat cereal	14.10	15.30

and concentrations (ppm) of the controls form a standard curve, and the sample ppm values of gliadin are interpolated from the curve based on their optical density values.

Test Kit Information

(**a**) *Kit name*.—Veratox® R5 Gliadin test kit.
(**b**) *Cat. No*.—8510.
(**c**) *Ordering information*.—*In the United States*.—Neogen Corp., 620 Lesher Pl, Lansing, MI 48912, phone: 517-372-9200, fax: 517-372-0108, www.neogen.com. *Outside the United States*.—Contact above for local distributor information.

Test Kit Reagents

(**a**) *Antibody-coated microwells (48)*.
(**b**) *Red-marked mixing wells (48)*.
(**c**) Yellow-labeled bottles of 1.2 mL each 0.0, 2.5, 10.0, 20.0, and 40.0 ppm gliadin standards.
(**d**) Two blue-labeled bottles of 4 mL enzyme-labeled antibody conjugate solution.
(**e**) One green-labeled bottle of 12.5 mL K-Blue substrate solution (tetramethylbenzidine).
(**f**) One red-labeled bottle of 12.5 mL Red Stop solution (0.0006% NaF + cresol red).
(**g**) One bottle of 40 mL 10 mM PBS-Tween washing reagent in a wide mouth bottle. Each bottle can prepare 1 L in distilled or deionized water (pH 7.4).
(**h**) One foil pouch sample diluent concentrate of 10 mM PBS dry powder containing enough powder to prepare 1 L of dilution buffer.
(**i**) Two cups of 50 g extraction additive.
(**j**) Plastic scoop to measure extraction additive.

Additional Supplies and Reagents

(Required but not included in the test kit).
(**a**) Gliadin renaturing cocktail solution for heat-processed samples (Neogen item No. 8515).
(**b**) Two 1 L bottles to prepare washing solution and sample extract dilution solution.
(**c**) Test tubes to perform sample extract dilution.
(**d**) Timer.
(**e**) Three reagent boats for 12-channel pipettor.
(**f**) Wash bottle.
(**g**) Paper towels or equivalent absorbent material.
(**h**) Waterproof marker.
(**i**) Distilled or deionized water.
(**j**) Laboratory grade ethanol (190 proof).

Apparatus

(**a**) Scale capable of weighing 0.25 ± 0.01 g.
(**b**) Microwell reader or strip reader with a 650 nm filter or equivalent with Neogen Veratox software.
(**c**) 5–300 µL 12-channel adjustable pipettor.
(**d**) 5–200 µL adjustable pipettor.
(**e**) Compatible tips for pipettors.
(**f**) Orbital rotator or shaker to hold 50 cc centrifuge tubes during extraction.
(**g**) Oven or water bath adjustable to 50°C if analyzing heat-processed samples.

Reference Material Used in Standard Preparation and Spiking

Material used for preparation of kit standards and spiking experiments was Gliadin G3375 from Sigma-Aldrich (St. Louis, MO) calibrated against the Prolamine Working Group standard; Zwickau, Germany (4). Spiking solution was prepared by weighing 25 mg of the above material using an analytical balance with 0.001 g precision and quantitatively transferring to a 50 mL polypropylene centrifuge tube. The material was then dissolved in 25 mL deionized water and vortexed for 2 min

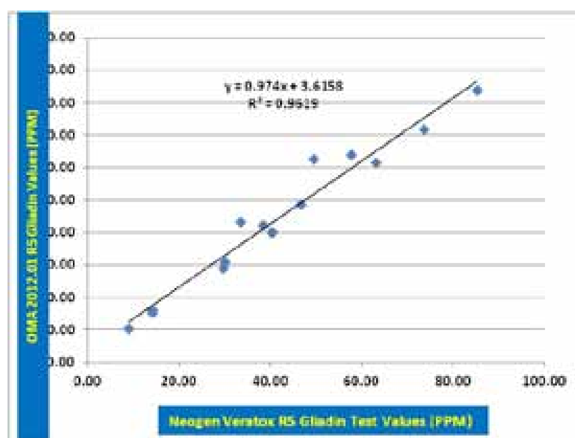

Figure 1. Scatter plot of methods comparison.

Table 3. Veratox R5 gliadin cross-reactivity

No.	Commodities	Result
1	Adzuki bean	Below LOD
2	Almond	Below LOD
3	Anasazi bean	Below LOD
4	Black eye pea	Below LOD
5	Black turtle bean	Below LOD
6	Bovine gelatin	Below LOD
7	Brazil nut	Below LOD
8	Brown rice	Below LOD
9	Buckwheat	Below LOD
10	Cashew	Below LOD
11	Chestnut	Below LOD
12	Chick pea	Below LOD
13	Cocoa	Below LOD
14	Coconut	Below LOD
15	Raw shrimp	Below LOD
16	Whole dried egg	Below LOD
17	Golden flaxseed	Below LOD
18	Great northern bean	Below LOD
19	Lentil	Below LOD
20	Green split peas	Below LOD
21	Hazelnut	Below LOD
22	Kidney bean	Below LOD
23	Lima bean	Below LOD
24	Macadamia nut	Below LOD
25	Ground pork	Below LOD
26	Ground beef	Below LOD
27	Ground chicken	Below LOD
28	Ground turkey	Below LOD
29	Navy beans	Below LOD
30	NFDM[a]	Below LOD
31	Oats	Below LOD
32	Peanut	Below LOD
33	Pecan	Below LOD
34	Pine nut	Below LOD
35	Pinto bean	Below LOD
36	Pistachio	Below LOD
37	Poppy seed	Below LOD
38	Porcine gelatin	Below LOD
39	Potato flour	Below LOD
40	Pumpkin seed	Below LOD
41	Medium grain rice	Below LOD
42	Sesame seed	Below LOD
43	Soy lecithin	Below LOD
44	Soy bean	Below LOD
45	Sunflower seed	Below LOD
46	Sorghum flour	Below LOD
47	Tapioca	Below LOD
48	Walnut	Below LOD
49	Whey	Below LOD
50	Corn syrup (light)	Below LOD
51	Wine (Merlot)	Below LOD
52	Yeast (active dry)	Below LOD
53	Cinnamon (ground)	Below LOD
54	Peppercorn	Below LOD
55	Parsley flakes	Below LOD
56	Black bean	Below LOD
57	Cranberry bean	Below LOD
58	Brown flaxseed	Below LOD
59	Chestnut flour	Below LOD
60	Amaranth	Below LOD
61	Corn	Below LOD
62	Teff	Below LOD
63	Rice	Below LOD
64	Oats	Below LOD
65	Millet	Below LOD
66	Wheat	100%
67	Barley	228%
68	Rye	243%
69	Spelt	210%
70	Triticale	180%
71	Kamut	100%

[a] NFDM = Nonfat dry milk.

for a final concentration of 1 mg/mL uniform suspension. This 1 mg/mL suspension was further diluted to 100 µg/mL with 2% carboxy methyl cellulose (CMC) solution containing 0.05% thimerosal and 0.25% bovine serum albumin in PBS pH 7.4. This solution was used for all spiking experiments (5).

Spiked samples were prepared by portioning finely ground gluten-free commodities into 0.25 g sample sizes in 50 cc polypropylene test tubes. Samples were then spiked directly with their respective ppm level of gliadin in the tube. Serial dilutions of spike solution were performed in CMC solution to ensure a constant spike volume of 25 µL was maintained for every level. Once spiked, blind-coded samples were held for 24 h at 4°C before analysis.

Incurred samples were prepared by portioning gluten-free bread mix (major ingredients include buckwheat and garbanzo

Table 4. Internal LOD, ppm

Replicate	Rice[a]	Oats	Sorghum	Corn	Buckwheat	Sausage	Cracker	Cereal
1	0.70	0.00	1.50	0.30	0.00	1.00	0.40	0.00
2	0.40	0.20	0.20	0.20	0.00	0.20	0.80	0.00
3	0.30	0.00	0.00	0.40	0.00	0.10	0.50	0.30
4	0.60	0.10	0.50	0.10	0.00	0.20	0.40	0.10
5	0.80	0.50	0.90	0.50	0.10	1.00	0.80	0.50
6	0.20	0.40	1.00	0.40	0.00	0.70	0.60	0.30
7	0.20	0.10	0.60	0.20	0.00	0.40	0.40	0.20
8	0.50	0.30	1.20	0.30	0.10	0.20	0.50	0.00
9	0.10	0.10	1.00	0.10	0.00	0.10	0.40	0.30
10	0.00	0.50	0.20	0.20	0.00	0.60	0.70	0.00
Mean	0.38	0.22	0.71	0.27	0.02	0.45	0.55	0.17
SD	0.27	0.19	0.49	0.13	0.04	0.35	0.16	0.18
Mean + 3.3 SD	1.26	0.86	2.32	0.71	0.16	1.62	1.09	0.75
Overall LOD = 1.10 ppm								

[a] All samples previously tested gluten-free.

bean flour) into 5–100 g batches. One was spiked with 10 mL 2% CMC solution only for a 0 ppm gliadin level. The remaining portions were spiked using above preparation with adjusted concentrations to ensure each respective level (5, 10, 20, and 40 ppm) were created by spiking 10 mL based on dry weight. Water was then added, and each batch was mixed according to the recipe and baked at 220°C for 22 min. Samples were then dried in a household food dehydrator, frozen, and ground to a fine particle size before they were portioned into 0.25 g sample size in 50 cc polypropylene centrifuge tubes and blind-coded. Samples were held for at 4°C awaiting analysis.

Sample Preparation and Extraction

All samples were blind-coded and stored at 2–8°C until analyzed.

(**a**) Prepare 80% ethanol extraction solution by combining eight parts ethanol with two parts (v/v) distilled water.

(**b**) Prepare sample extract dilution solution (PBS) by dissolving the contents of the packet in 1 L distilled water.

(**c**) Weigh out 0.25 g sample into a 50 cc screw cap centrifuge tube.

(**d**) Add 2.5 mL renaturing cocktail solution.

(**e**) If samples contain buckwheat, chestnut flour, tannins, or phenolic compounds, like chocolate, coffee or cocoa, add one level scoop of extraction additive. For all other commodity types, do not add extraction additive.

(**f**) Cap and vortex 30 s to homogenize cocktail and sample.

(**g**) Incubate 40 min at 50°C (water bath or oven).

(**h**) Remove samples and let cool 5–10 min.

(**i**) Add 7.5 mL 80% ethanol and vortex again for 10–20 s.

(**j**) Shake (150–200 rpm) for 1 h at room temperature on a rotator (tube on its side).

(**k**) Centrifuge sample (if material does not readily settle) for 10 min at ≥2500 rpm.

(**l**) Dilute the sample 1:12.5 with PBS (200 µL sample + 2.3 mL PBS).

(**m**) Samples are ready for analysis.

Procedural Notes

(**a**) *Wash buffer*.—Prepare the wash buffer solution by diluting the wash buffer concentrate in 960 mL distilled or deionized water in an empty 1 L container. Ensure transfer of all concentrate by rinsing the bottle several times with water, and swirl to ensure thorough mixing.

(**b**) *Substrate*.—K-Blue substrate is ready for use. The substrate should be clear to light blue; discard if it has turned dark blue. Only pour the needed volume of substrate into a reagent boat. Do not return unused substrate to the bottle. Cover the reagent boat to keep the substrate protected from light until needed.

(**c**) *Antibody wells*.—Keep wells sealed in the foil pouch until needed. Remove wells from the foil pouch only after samples are extracted and the test procedure is set to begin.

Precautions

(**a**) Ethanol solution is highly flammable. Keep container

Table 5. Temperature robustness

	Samples, ppm		
Temperature, °C	0	5	10
Run No. 1			
18	0.20	4.70	9.70
24	0.00	4.90	9.50
30	0.00	4.60	9.70
Run No. 2			
18	0.60	4.50	9.90
24	0.00	4.50	9.40
30	0.10	4.90	9.50

Table 6. Robustness of volume

	Samples, ppm		
Volume, µL	0	5	10
Run No. 1			
95	0.00	5.30	9.60
100	0.00	4.90	10.60
105	0.00	4.30	8.70
Run No. 2			
95	0.00	5.20	9.90
100	0.00	5.70	9.60
105	0.00	4.40	8.80

Table 7. Robustness of shaking effects

	Samples, ppm		
Shaking, s	0	5	10
Run No. 1			
No shake (0)	0.40	4.80	9.70
10	0.00	4.10	9.20
20	0.00	4.60	9.00
Run No. 2			
No shake (0)	0.40	4.70	9.50
10	0.00	4.50	8.50
20	0.00	4.60	8.10

Table 8. Robustness of assay time

	Samples, ppm		
Run 1 time, min	0	5	10
10:10:10	0.00	5.40	10.50
8:8:8	0.00	5.60	9.60
12:12:12	0.00	6.35	9.60
8:10:12	0.50	5.50	8.60
Run 2 time, min	0.00	5.00	10.00
10:10:10	0.20	4.90	10.40
8:8:8	0.00	5.30	10.10
12:12:12	0.00	5.10	9.90
8:10:12	0.00	5.40	9.00

tightly closed, and keep away from heat, sparks, open flame, and those smoking. It is toxic if swallowed or if vapor is inhaled. Avoid contact with skin.

(**b**) Components of Veratox R5 Gliadin test kit, such as controls and extraction additive, may contain one or more of the following potentially allergic materials: gluten, casein, almond protein, and soy protein. If allergic to any of these compounds, be cautious when using this product.

(**c**) Store test kit between 2–8°C (35–46°F) when not in use. Do not freeze test kits, and avoid prolonged storage at ambient temperatures.

(**d**) Bring kits to ambient temperature (18–30°C, 64–86°F) prior to use.

(**e**) Do not use kit components beyond expiration date.

(**f**) Do not mix reagents from one kit lot with reagents from a different lot.

(**g**) Use clean pipet tips and glassware for each sample to avoid cross-contamination. Thoroughly wash all glassware between samples.

Analysis

(**a**) Remove one red-marked mixing well for each sample to be tested, plus five for controls, and place in the well holder (strips consist of 12 wells and are breakable).

(**b**) Remove an equal number antibody-coated wells. Return antibody wells that will not be used immediately to the foil pack with desiccant. A minimum of seven wells are necessary for a single sample (strips consist of 12 wells and are breakable).

(**c**) Using a new pipet tip for each, transfer 150 µL of controls and sample extracts to the red-marked transfer wells (transfer wells are used to stage the assay to eliminate end-to-end effect caused by differing pipetting proficiency).

(**d**) Place tips on the 12-channel pipettor and transfer 100 µL controls and sample extracts to the antibody-coated wells. Mix for 20 s by sliding the well holder back and forth on a flat surface.

(**e**) Incubate microwells 10 min at ambient

Table 9. Lot-to-lot variability

	Lot No.		
Lot-to-Lot	159002 6/8/11	159005 9/30/11	159008 11/27/11
Level, ppm	12 days to expiry, ppm	124 days to expiry, ppm	181 days to expiry, ppm
0	0.00	0.00	0.00
0	0.00	0.00	0.50
0	0.00	0.10	0.00
5	4.60	4.80	4.70
5	4.80	5.20	4.60
5	5.10	5.40	4.50
10	9.70	10.50	10.40
10	10.10	9.90	10.20
10	9.70	10.20	10.20
R^2	0.995	0.994	0.985

Table 10. Gluten-free rice cereal accuracy and precision from independent laboratory

Target concn, ppm	ID No.	Neogen sample ID No.	Dilution factor	Final result, ppm		Recovery, %	
				Replicate			
				1	2	1	2
0.0	1	1887	1.0	0.8	0.0	NA[a]	NA
	2	3172	1.0	0.3	0.2	NA	NA
	3	4047	1.0	2.0	0.1	NA	NA
	4	2994	1.0	0.8	0.5	NA	NA
	5	181	1.0	0.3	0.2	NA	NA
	1n	506	1.0	0.3	1.44	NA	NA
	2n	2809	1.0	0.0	0.16	NA	NA
	3n	3173	1.0	0.0	0.22	NA	NA
	4n	3277	1.0	0.0	0.0	NA	NA
	5n	4879	1.0	0.8	0.0	NA	NA
5.0	6	612	1.0	6.9	6.8	138	136
	7	2594	1.0	6.6	6.6	130	132
	8	1024	1.0	5.6	5.8	112	116
	9	2386	1.0	6.9	7.5	138	150
	10	4500	1.0	5.7	6.8	114	136
	6n	1134	1.0	5.2	4.7	104	94
	7n	1415	1.0	5.0	4.7	100	94
	8n	2022	1.0	4.9	4.6	98	92
	9n	2803	1.0	5.0	5.4	100	108
	10n	4694	1.0	4.9	5.2	98	104
10.0	11	3026	1.0	16.1	15.9	161	159
	12	1975	1.0	10.0	9.2	100	92
	13	1482	1.0	8.5	8.1	85	81
	14	2202	1.0	9.9	8.6	99	86
	15	1581	1.0	8.3	8.4	83	84
	11n	879	1.0	8.7	7.5	87	75
	12n	1398	1.0	9.0	8.7	90	87
	13n	2882	1.0	7,4	9.8	74	98
	14n	4019	1.0	8.2	9.2	82	92
	15n	4529	1.0	7.6	9.1	76	91
20.0	16	1472	1.0	21.4	20.3	107	101.5
	17	1082	1.0	24.8	24.4	124	121.5
	18	1073	1.0	29.0	30.2	145	151
	19	1512	1.0	14.9	14.5	74.5	72.5
	20	4656	1.0	12.7	12.2	63.5	61
	16n	313	1.0	23.0	18.4	115	92
	17n	696	1.0	22.0	21.6	110	108
	18n	1514	1.0	22.9	20.7	115	104
	19n	1725	1.0	21.2	14.02	106	70.1
	20n	2779	1.0	21.7	22.1	109	110.5
40.0	21	375	2.0	26.2	27.8	65.5	69.5
	22	1475	1.0	32.3	32.6	80.8	81.5
	23	265	2.0	32.6	33.0	81.5	82.5
	24	3371	2.0	33.8	31.4	84.5	78
	25	3757	2.0	29.2	28.8	73	72
	21n	1644	1.0	46.7	52.6	116.8	131.5
	22n	2335	1.0	40.1	50.8	100.2	127
	23n	2437	1.0	51.9	43.8	129.7	109.5
	24n	3274	1.0	47.5	48.9	118.8	122.3
	25n	4847	1.0	46.0	49.0	115	122.5

[a] NA = Not applicable.

temperature (18–30°C, 64–86°F). Discard the red-marked transfer wells.

(**f**) Empty the contents of the wells into a sink. With a wash bottle, fill each antibody well with the wash buffer solution and dump out. Repeat the washing five times, then turn the wells upside down and tap out on a paper towel until all washing solution is removed.

(**g**) Pour the needed volume of conjugate from the blue-labeled bottle into a clean reagent boat.

(**h**) Using the 12-channel pipettor and new tips, transfer 100 µL conjugate into all the wells and mix for 20 s by sliding the well holder back and forth on a flat surface.

(**i**) Incubate for 10 min at ambient temperature.

(**j**) Wash all wells with the wash buffer solution as described previously.

(**k**) Pour the needed volume of substrate solution from the green-labeled bottle into a clean reagent boat.

(**l**) Place new tips on the 12-channel pipettor, transfer 100 µL substrate into each well, and mix for 20 s.

(**m**) Incubate for 10 min at ambient temperature.

(**n**) Pour the needed volume of Red Stop solution from the red-labeled bottle into a clean reagent boat.

(**o**) Place new tips on the 12-channel pipettor, transfer 100 µL Red Stop into each well, and mix for 20 s.

(**p**) Wipe the bottom of the microwells and read in a microwell reader with a 650 nm filter.

(**q**) Interpret the test's results using Neogen's Stat Fax microwell reader, or an equivalent strip reader. If using a strip reader, calculate the results using Neogen's Veratox software for Windows.

Interpretation of Results

Results between 2.5 and 40 ppm are quantitative. Results greater than 40 ppm should be diluted for proper quantitation. The standards are prepared from wheat gliadin and are calculated as ppm gliadin. Approximately 50% of the gluten is available as gliadin. Therefore, to calculate the gluten value of the samples, multiply the ppm gliadin results by 2 if desired. For the purposes of this validation study, all results will be reported as gliadin even if they are below the lowest calibrator.

Table 11. Cooked hamburger accuracy and precision from independent laboratory

				Final result, ppm		Recovery, %	
				Replicate			
Target concn, ppm	Q Labs (QL) ID No.	Neogen sample ID No.	Dilution factor	1	2	1	2
0.0	1	4481	1.0	0.0	0.0	NA[a]	NA
	2	3893	1.0	0.0	0.0	NA	NA
	3	3517	1.0	0.0	0.0	NA	NA
	4	2762	1.0	0.0	0.0	NA	NA
	5	1724	1.0	0.0	0.0	NA	NA
	1n	747	1.0	0.8	0.5	NA	NA
	2n	3885	1.0	0.2	0.5	NA	NA
	3n	3999	1.0	0.7	0.2	NA	NA
	4n	1663	1.0	0.6	0.6	NA	NA
	5n	4440	1.0	0.5	0.4	NA	NA
5.0	6	1429	1.0	9.3	9.3	186	186
	7	1649	1.0	9.3	8.5	186	170
	8	4183	1.0	9.5	9.4	190	188
	9	2351	1.0	9.6	8.8	192	176
	10	3197	1.0	7.5	7.9	150	158
	6n	269	1.0	4.7	5.1	94	102
	7n	1481	1.0	4.9	5.1	98	102
	8n	2007	1.0	4.5	4.7	90	94
	9n	939	1.0	5.1	5.1	102	102
	10n	691	1.0	4.9	4.8	98	96
10.0	11	4137	1.0	11.9	11.9	119	119
	12	3710	1.0	11.8	12.3	118	123
	13	4731	1.0	13.5	12.7	135	127
	14	1888	1.0	12.0	12.4	120	124
	15	3675	1.0	12.3	12.4	123	124
	11n	1670	1.0	8.1	7.3	81	73
	12n	1370	1.0	8.3	9.4	83	94
	13n	4360	1.0	7.3	8.8	73	88
	14n	4353	1.0	7.5	8.0	75	80
	15n	3442	1.0	7.5	7.9	75	79
20.0	16	3435	1.0	18.7	19.8	91	99
	17	4226	1.0	19.2	19.4	96.5	96.5
	18	86	1.0	19.0	19.4	95	96.5
	19	1905	1.0	17.5	18.3	87.5	91.5
	20	1895	1.0	20.2	19.8	101	99
	16n	460	1.0	23.2	20.9	116	104.5
	17n	901	1.0	21.9	23.4	109.5	117
	18n	3823	1.0	21.7	20.8	108.5	104
	19n	4891	1.0	21.0	21.1	105	105.5
	20n	1711	1.0	25.2	23.1	126	115.5
40.0	21	4464	1.0	36.8	33.5	92	83.8
	22	1423	1.0	30.0	28.5	75	71.3
	23	2680	1.0	29.5	27.3	73.8	68.3
	24	1774	1.0	30.8	29.8	77	74.5
	25	4664	1.0	32.0	26.4	80	66
	21n	1797	1.0	32.8	33.5	82	83.8
	22n	4076	1.0	34.7	34.2	86.8	85.5
	23n	4851	2.0	35.1	35,9	87.8	89.8
	24n	21	2.0	33.7	34.5	84.3	86.3
	25n	1664	2.0	31.1	31.7	77.8	79.3

[a] NA = Not applicable.

Internal Validation Studies

All analyses were generated from one kit lot unless specified.

Spike and Recovery (Repeatability and Recovery)

Blind-coded samples were prepared from each of eight gluten-free products spiked at various levels (unspiked, 5.0, 10.0, and 25.0 ppm) as described in *Reference Material Used in Standard Preparation and Spiking* section of this document. Each sample was extracted and independently analyzed by three individual technicians in the same laboratory, using the same reader. Each technician extracted and analyzed a separate sample set.

(**a**) *Testing*.—The above preparations were tested following procedure set forth in *Sample Preparation and Extraction, Procedural Notes, Precautions, Analysis,* and *Interpretation of Results* sections of this document.

(**b**) *Results*.—The evaluation of sample extracts for recovery indicates an average of 113.70% recovery at 5.0 ppm, 111.60% recovery at 10 ppm, and 106.70% recovery at 25 ppm (Table 1) across all matrixes. An overall 110.70% recovery was observed across all levels. The evaluation of the same sample extracts for repeatability indicates a 3.40% RSD at 5.0 ppm, a 4.00% RSD at 10.0 ppm, and an 8.10% RSD at 25.0 ppm across all matrixes. An overall 5.20% RSD was observed across all levels and matrixes (Table 1).

Comparison to Existing Methods

Naturally incurred samples were sourced at local stores and used when found to contain measurable gliadin levels. Duplicate samples were blind-coded and analyzed by the candidate Veratox ELISA method and OMA **2012.01**. All samples analyzed in triplicate and means were compared.

(**a**) *Testing*.—The above preparations were tested following procedures set forth in *Sample Preparation and Extraction, Procedural Notes, Precautions, Analysis,* and *Interpretation of Results* sections of this document for the Neogen method.

(**b**) *Results*.—Data indicate 0.961 correlation between the Veratox ELISA method and the OMA method **2012.01**

Table 12. Gluten-free bread accuracy and precision from independent laboratory

Target concn, ppm	Q Labs (QL) ID No.	Neogen sample ID No.	Dilution factor	Final result, ppm Replicate 1	Final result, ppm Replicate 2	Recovery, % Replicate 1	Recovery, % Replicate 2
0.0	1	1061	1.0	0.0	0.0	NA[a]	NA
	2	4661	1.0	0.0	0.8	NA	NA
	3	1338	1.0	0.0	0.0	NA	NA
	4	209	1.0	0.0	0.0	NA	NA
	5	1286	1.0	0.0	0.0	NA	NA
	1n	903	1.0	0.0	0.0	NA	NA
	2n	676	1.0	0.0	0.0	NA	NA
	3n	2170	1.0	0.0	0.0	NA	NA
	4n	2425	1.0	0.0	0.0	NA	NA
	5n	2633	1.0	0.0	0.0	NA	NA
5.0	6	4588	1.0	8.7	8.8	174	176
	7	3876	1.0	9.9	9.7	198	194
	8	1671	1.0	8.7	8.7	174	174
	9	1236	1.0	8.3	8.6	166	172
	10	3475	1.0	9.3	9.0	186	180
	6n	125	1.0	7.1	7.0	142	140
	7n	557	1.0	7.3	7.0	146	140
	8n	3470	1.0	7.3	6.4	146	128
	9n	4827	1.0	4.5	5.6	90	112
	10n	2675	1.0	5.5	5.4	110	108
10.0	11	450	1.0	10.7	10.5	107	105
	12	4175	1.0	12.9	13.5	129	135
	13	4845	1.0	13.0	11.9	130	119
	14	4004	1.0	11.6	11.2	116	112
	15	2674	1.0	10.3	10.3	103	103
	11n	348	1.0	9.8	9.5	98	95
	12n	1667	1.0	9.1	9.7	91	97
	13n	2405	1.0	9.8	10.2	98	102
	14n	2417	1.0	9.5	9.7	95	97
	15n	3148	1.0	10.3	10.5	103	105
20.0	16	3193	1.0	20.9	19.7	105	99
	17	1665	1.0	19.1	18.3	96	92
	18	445	1.0	20.1	19.9	101	100
	19	964	1.0	18.4	16.8	92	84
	20	369	1.0	17.0	16.0	85	80
	16n	278	1.0	20.8	19.5	104	97.5
	17n	1117	1.0	24.7	23.3	123.5	116.5
	18n	1442	1.0	24.1	23.8	120.5	119
	19n	2115	1.0	23.3	22.5	116.5	112.5
	20n	4069	1.0	21.3	18.8	106.5	94
40.0	21	2177	1.0	31.8	30.1	80	75
	22	2343	1.0	37.7	33.8	94	85
	23	4287	1.0	31.0	31.8	78	80
	24	4128	1.0	28.5	27.9	71	70
	25	3436	1.0	30.6	31.0	77	78
	21n	878	1.0	36.4	34.5	91	86.3
	22n	2853	1.0	34.9	35.7	87.3	89.3
	23n	3224	1.0	25.2	31.9	63	79.8
	24n	3514	1.0	34.7	35.8	86.7	89.5
	25n	2675	1.0	37.2	39.2	93	98

[a] NA = Not applicable.

(Table 2). A scatter plot shows a linear relationship with a slope of 0.974 (Figure 1).

Cross-Reactivity

Data were generated quantifying cross-reactivity for the Veratox kit with various gluten-containing and nongluten-containing foods.

(**a**) *Preparation of cross-reactors*.—Each potential cross-reactive food was purchased at a local store and extracted per kit instructions. Gluten-containing ingredients reporting greater than 40 ppm were diluted to fit into the scale of test kit and multiplied by the necessary dilution factor.

(**b**) *Testing*.—The above preparations were tested following procedures set forth in *Sample Preparation and Extraction, Procedural Notes, Precautions, Analysis,* and *Interpretation of Results* sections of this document for the Neogen method.

(**c**) *Results*.—Cross-reactivity was not observed on any of the gluten-free foods, as all reported values were below the LOD. All other prolamins were detected. Percent recoveries were all calculated against wheat (Table 3).

LOD

Data are submitted showing the LOD of one sample of each of the gluten-free foods tested 10 times. The LOD is expressed as the mean value of the negative sample determination plus 3.3 SDs.

(**a**) *Testing*.—The above preparations were tested following procedure set forth in *Sample Preparation and Extraction, Procedural Notes, Precautions, Analysis,* and *Interpretation of Results* sections of this document.

(**b**) *Results*.—LOD was determined to be 1.26 ppm (rice), 0.86 ppm (oats), 0.1 ppm (buckwheat), 2.32 ppm (sorghum), 0.71 ppm (corn), 1.62 ppm (sausage), 1.09 ppm (crackers), and 0.75 ppm (cereal) by ELISA, with a mean LOD of 1.1 ppm across all matrixes tested (Table 4).

Ruggedness Testing

Data are included demonstrating ruggedness based on assay

Table 13. Rice flour accuracy and precision from independent laboratory

Target concn, ppm	Q Labs (QL) ID No.	Neogen sample ID No.	Dilution factor	Final result, ppm		Recovery, %	
				Replicate			
				1	2	1	2
0.0	1	2518	1.0	0.0	0.0	NA	NA
	2	457	1.0	0.0	0.0	NA	NA
	3	794	1.0	0.0	0.2	NA	NA
	4	4450	1.0	1.3	0.0	NA	NA
	5	4123	1.0	0.3	0.2	NA	NA
	1n	249	1.0	0.1	0.1	NA	NA
	2n	4163	1.0	0.6	0.3	NA	NA
	3n	4976	1.0	0.2	0.2	NA	NA
	4n	4361	1.0	0.0	0.0	NA	NA
	5n	602	1.0	0.4	0.3	NA	NA
5.0	6	937	1.0	6.6	7.3	132	146
	7	898	1.0	9.4	10.0	188	200
	8	2948	1.0	6.4	6.4	128	128
	9	1038	1.0	7.7	8.1	154	162
	10	1475	1.0	8.0	6.9	160	138
	6n	1737	1.0	4.8	4.6	96	92
	7n	4051	1.0	5,1	4.1	102	82
	8n	4983	1.0	4.1	4.8	82	90
	9n	4460	1.0	5.4	4.6	108	92
	10n	3117	1.0	4.3	4.5	86	90
10.0	11	1490	1.0	11.8	11.1	118	111
	12	1539	1.0	11.3	11.1	113	111
	13	1426	1.0	11.1	10.6	111	106
	14	3270	1.0	11.3	11.0	113	110
	15	3830	1.0	12.1	12.9	121	129
	11n	2868	1.0	7.7	7.2	77	72
	12n	3739	1.0	8.8	8.8	88	88
	13n	2709	1.0	7.3	8.9	73	89
	14n	2895	1.0	7.7	7.9	77	79
	15n	2846	1.0	8.5	8.9	85	89
20.0	16	2846	1.0	16.2	15.6	81	78
	17	3135	1.0	21.2	20.4	106	102
	18	3683	1.0	19.0	19.8	95	99
	19	1693	1.0	19.5	17.3	97.5	86.5
	20	2340	1.0	19.5	19.9	97.5	99.5
	16n	3830	1.0	23.9	18.8	119.5	94
	17n	1019	1.0	21.5	23.3	107.8	116.5
	18n	2805	1.0	22.5	22.8	112.5	114
	19n	4591	1.0	24.0	22.1	120	110.5
	20n	1777	1.0	23.2	21.6	116	108
40.0	21	4315	1.0	36.8	40.8	92	102
	22	2447	1.0	40.4	37.1	101	92.8
	23	3851	1.0	37.4	38.1	93.5	95.2
	24	2459	1.0	33.6	37.1	84	92.8
	25	1105	1.0	49.4	55.0	123	137.5
	21n	3165	2.0	37.2	38.7	93	96.3
	22n	1897	1.0	36.8	38.4	92	96
	23n	178	1.0	38.9	41.1	97.3	102.8
	24n	1600	2.0	41.2	23.1	103	107.8
	25n	28	2.0	38.5	37.9	96.3	94.8

[a] NA = Not applicable.

of three spiked samples of gluten-free corn (0, 5, and 10 ppm) tested over three conditions each, over four different parameters each, and over 2 days. The three conditions represent a low, medium, and high value of each parameter. Parameters include temperature, shaking time, reagent volume, and incubation time. Gluten-free corn was spiked using reference material described in *Reference Material Used in Standard Preparation and Spiking* section of this document. All samples were blind-coded.

(**a**) *Temperature*.—All parameters of the kit were used as indicated in *Sample Preparation and Extraction, Procedural Notes, Precautions, Analysis,* and *Interpretation of Results* sections of this document, with the exception of assay temperature, which was intentionally altered to include 18, 24, and 30°C. These temperature differences appear to have little effect on results when compared to the optimal temperature of 24°C (Table 5).

(**b**) *Reagent volume*.—All parameters of the kit were used as indicated in *Sample Preparation and Extraction, Procedural Notes, Precautions, Analysis,* and *Interpretation of Results* sections of this document, with the exception of reagent volume for all steps, which was intentionally altered to include 95, 100, and 105 µL. Data indicate these differences in volume have little effect on results when compared to the optimum volume of 100 µL (Table 6).

(**c**) *Shaking time*.—All parameters of the kit were used as indicated in *Sample Preparation and Extraction, Procedural Notes, Precautions, Analysis,* and *Interpretation of Results* sections of this document, with the exception of shaking time for both competition and substrate steps, which was intentionally altered to include no shaking, 10 s, and 20 s. These differences appear to have little effect on results (Table 7).

(**d**) *Incubation time*.—All parameters of the kit were used as indicated in *Sample Preparation and Extraction, Procedural Notes, Precautions, Analysis,* and *Interpretation of Results* sections of this document, with the exception of incubation time for all steps, which was intentionally altered to include 8 min, 12 min, and a combination of 8, 10, and 12 min. These differences appear to have little effect on results. While little effect was observed, an optimum timing of 10 min/step is still recommended (Table 8).

Table 14. Data summary of gluten-free and spiked rice cereal

Spike levels, ppm	0	5	10	20	40
Mean, ppm	0.41	5.74	9.41	20.6	39.25
Mean recovery, %	NA[a]	114.70	94.10	103.10	98.10
Repeatability SD	0.53	0.92	2.37	4.94	9.26
Repeatability RSD, %	NA	16.03	25.19	24.18	23.59
No. of replicates with acceptable recovery[b]	NA	10/20	18/20	19/20	20/20
LOD, ppm	2.16				
LOQ, ppm	5.71				
Mean RSD, %	22.50				
Mean recovery, %	102.60				

[a] NA = Not applicable.

[b] The actual percentages are presented in Table 10.

Table 16. Data summary of gluten-free and gluten-incurred bread

Spike levels, ppm	0	5	10	20	40
Mean, ppm	0.04	7.64	10.70	20.42	32.99
Mean recovery, %	NA[a]	151.68	107.11	102.39	82.99
Repeatability SD	0.18	1.56	1.26	2.56	3.61
Repeatability RSD, %	NA	20.42	11.78	12.54	10.94
No. of replicates with acceptable recovery[b]	NA	10/20	20/20	20/20	20/20
LOD, ppm	0.63				
LOQ, ppm	1.84				
Mean RSD, %	13.92	13.92			
Mean Recovery, %	110.65	110.65			

[a] NA = Not applicable.

[b] The actual percentages are presented in Table 12.

Lot-to-Lot Variability and Stability

Lot-to-lot variability is assessed by testing samples of spiked gluten-free rice at 3 levels (0, 5, and 10 ppm). Results were taken from a standard curve. Assays were performed in triplicate for each of three different kit lots. The three kit lots represented reagents at the beginning, middle, and end of usable shelf life.

(**a**) *Testing*.—The above preparations were tested following the procedure set forth in *Sample Preparation and Extraction, Procedural Notes, Precautions, Analysis,* and *Interpretation of Results* sections of this document.

(**b**) *Results*.—Results generated from each lot of kits were found to be comparable (Table 9).

Independent Laboratory Validation

Matrixes

The matrixes for analysis were rice flour, gluten-free bread, gluten-free rice cereal, and cooked hamburger. Given the difficulty in preparing homogeneous incurred and bulk spiked samples, one matrix (gluten-free bread) was selected for incurred sample preparation, and the remaining three matrixes were individually spiked with a liquid suspension of wheat flour described below. All samples were prepared as indicated in *Reference Material Used in Standard Preparation and Spiking* sections of this document.

Table 15. Data summary of gluten-free and spiked cooked hamburger

Spike levels, ppm	0	5	10	20	40
Mean, ppm	0.25	6.90	10.17	20.68	32.09
Mean recovery, %	NA[a]	138.00	101.65	103.25	80.25
Repeatability SD	0.29	2.13	2.29	1.96	2.88
Repeatability RSD, %	NA	30.87	22.52	9.48	8.97
No. of replicates with acceptable recovery[b]	NA	11/20	20/20	20/20	20/20
LOD, ppm	1.21				
LOQ, ppm	3.15				
Mean RSD, %	17.96				
Mean recovery, %	105.79				

[a] NA = Not applicable.

[b] The actual percentages are presented in Table 11.

All samples were blind-coded and shipped under cold packs overnight for analysis by the independent laboratory on the day of arrival. A concurrent blind sample set was analyzed by Neogen in parallel. Levels and replicates were modeled after harmonized guidelines for allergen ELISA validation. Samples for spiking were ground in a household blender to a fine particle size and stored at –20°C awaiting study. All samples analyzed as described in *Analysis* section.

Five replicates of each level/matrix combination were randomly coded and sent overnight to the independent laboratory packed on ice packs and analyzed the day they arrived. Five replicates × five levels resulted in 25 test portions per matrix. One matrix was shipped each day for 4 consecutive days; each extraction was performed directly out of the tube provided.

All data above 0.0 ppm were reported for the purposes of the validation study. Due to the Neogen calculation algorithm, it is not possible to produce negative (less than 0 ppm) results. All 100 extractions were analyzed in duplicate for a total of 200 data points; a corresponding set of 200 data points was generated by Neogen on the same samples. While duplicate sample analysis is not a requirement of the method, it was requested in this case to increase the number of data points generated for statistical analysis.

Data Analysis

All results were tabulated and recoveries were determined, comparing to target levels to ensure that all results showed an acceptable recovery, ranging from 50 to 150% per *J. AOAC Int.* (6) Validation Procedures for Quantitative Food Allergen ELISA Methods: Community Guidance and Best Practices.

LOD was calculated for each matrix using AOAC/ISO 5725

Table 17. Data summary of gluten-free and spiked rice flour

Spike levels, ppm	0	5	10	20	40
Mean, ppm	0.21	6.21	9.80	20.61	38.88
Mean recovery, %	NA[a]	122.80	98.00	103.04	99.66
Repeatability SD	0.31	1.84	1.80	2.45	6.01
Repeatability RSD, %	NA	29.63	18.37	11.89	15.46
No. of replicates with acceptable recovery[b]	NA	15/20	20/20	20/20	20/20
LOD, ppm	1.23				
LOQ, ppm	3.31				
Mean RSD, %	18.84				
Mean recovery, %	105.87				

[a] NA = Not applicable.

[b] The actual percentages are presented in Table 13.

Standard by the basic formula of 3.3 × SD of a minimum of 10 negative samples (7).

LOQ was calculated for each matrix by AOAC/ISO 5725 Standard by the basic formula of 10 × SD of a minimum of 10 negative samples. Repeatability SD, reproducibility SD, repeatability RSD, and reproducibility RSD were calculated using AOAC/ISO 5725 Standard.

Results

Both independent laboratory data and duplicate Neogen data are shown in Tables 10–17. Neogen data points are differentiated by having the letter "n" following each sample ID. Mean RSD was 22.25% for breakfast cereal, 17.96% for cooked hamburger, 13.92% for bread, and 18.84% for rice flour across all levels tested.

Mean recovery was 102.6% for breakfast cereal, 105.79% for cooked hamburger, 110.65% for bread, and 105.87% for rice flour across all levels tested.

LOD was 2.16 ppm for breakfast cereal, 1.21 ppm for cooked hamburger, 0.63 ppm for bread, and 1.23 ppm for rice flour.

Conclusions

The data show a slight overestimation of gliadin at the 5 ppm level. However, all other levels meet performance claims for recovery and repeatability in accordance with the harmonized protocol for allergen methods validation. Method users should be aware of the potential to overestimate values in the 5 ppm range.

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