

Validation of the AgraStrip[®] Gluten G12 Test Kit

Performance Tested MethodSM 061403

Abstract

Romer Labs[®], Inc. developed an immunochromatographic lateral flow assay for the qualitative detection of gluten in raw ingredients, processed foods, finished food products, and environmental surfaces, using the G12 antibody developed by Belén Morón. The G12 antibody targets a 33-mer peptide which is resistant to enzymatic digestion and heat denaturation, as well as being the fragment of the gliadin protein to which celiac disease sufferers react, making it a reliable analytical marker. This study was performed to validate the AgraStrip[®] Gluten G12 assay method under the guidance of the AOAC *Performance Tested Methods*SM (PTM) program against AOAC *Official Method of Analysis*SM 2012.01 in rice flour, bread, cookie, ice cream, and chocolate matrixes spiked with either purified gliadin or wheat gluten standard at 0, 3, 8, 15, and 25 ppm concentrations and tested at the 5, 10, and 20 ppm assay thresholds, as well as on environmental surfaces. Stability, robustness, variation, and lot consistency studies were performed by spiking wheat gluten into a rice flour matrix at 0 and 15 ppm concentrations. The AgraStrip Gluten G12 assay was rigorously evaluated during this study and demonstrates its suitability as an AOAC PTM-certified gluten detection method.

Participants

METHOD AUTHORS

SCOTT RADCLIFFE, MEREDITH SUTZKO, ZHENG JIANG, DENISE FREITAG, CHRISTY SWOBODA, LUKAS FRANK, and ADRIAN ROGERS

Romer Labs, Technopark 1, 3430 Tulln, Austria

SUBMITTING COMPANY

Romer Labs, Technopark 1, 3430 Tulln, Austria

INDEPENDENT LABORATORY

Q Laboratories, Inc., 1400 Harrison Ave, Cincinnati, OH 45214

REVIEWERS

TODD MARROW

University of Guelph, Laboratory Services Division, 95 Stone Rd W, Guelph, Ontario N1H-8J7, Canada

MARY TRUCKSESS

Mycotoxin Consulting, 2745 Clarkes Landing Dr, Oakton, VA 22124

BERT POPPING

Eurofins Scientific, Inc., Jesteburgerstr. 8, 21218 Seevetal, Germany

Scope of Method

The AgraStrip[®] Gluten G12 test kit antibody, developed by Morón et al. (1, 2), specifically detects the immunotoxic 33-mer amino acid sequence of gliadin, toward which celiac patients have an autoimmune reaction, and cross-reacts to the homologous immunotoxic fragments of rye secalin and barley hordein. Additionally, the G12 antibody detects a homologous fragment in certain varieties of oats. The assay LOD for gluten in food products is 5 ppm (4 µg/25 cm² on surfaces), with extract cutoff dilutions set at 5, 10, and 20 ppm to comply with standards set by the Celiac Support Association (3), Gluten-Free Certification Organization (4), Gluten Free Certification Program (5), and Codex Alimentarius Standard 118-1979 (6).

Definitions

Probability of detection (POD).—The proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration. POD is concentration-dependent. The POD estimate is calculated as the number of positive outcomes divided by the total number of trials.

Introduction

Gluten describes the main group of proteins in wheat, rye, and barley, and consists of prolamins (in wheat: gliadin, rye: secalin, barley: hordein) and glutelins (in wheat: glutenin), occurring in the same ratio. Due to its physicochemical characteristics, gluten is used in food products as a binder, to help dough rise, and to give dough a more appetizing texture (7).

Celiac disease is an autoimmune disorder of the small intestine resulting in malabsorption and inflammation. In persons who are genetically susceptible, it is caused by an immune reaction to gliadin, which cannot be enzymatically degraded in the intestine due to its high proline and glutamine content. The only effective treatment is a lifelong gluten-free diet (7).

According to Codex Alimentarius Standard 118-1979, “gluten-free” products must comply with gluten levels (including prolamin fractions from rye, barley and oats) below 20 mg/kg (ppm) and “foods specially processed to reduce gluten content” must comply with levels between 20 and 100 mg/kg (ppm); (6). Utilization of a reliable and accurate screening method for gluten-free ingredients, foods, and processing equipment will ensure safety of food products for celiac sufferers.

Submitted for publication July 22, 2014.

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.

Corresponding author's e-mail: scott.radcliffe@romerlabs.com

DOI: 10.5740/jaoacint.14-143

Principle of the Method

The AgraStrip Gluten G12 test kit is a lateral flow immunochromatographic assay for the detection of gluten. The test kit is designed to detect the presence of gluten in foods with varying compositions and levels of processing, from raw foods/ingredients to finished product testing. Additionally, the test kit is designed to detect the presence of gluten on surfaces through swab testing.

General Information

The test consists of a 1 min, room temperature extraction of 0.2 g of a sample, or a swab of a 25 cm² surface (rinse water testing does not require an extraction step). Three drops of the extract are then passed through a filter tip into a vial filled with diluent to one of three lines demarcating test cutoffs at 5, 10, or 20 ppm gluten. The detection step is based on the formation of a complex between the immunotoxic fractions of gluten in the sample with colored antibody conjugates (monoclonal anti-gliadin 33-mer antibody/red-colored microsphere) previously fixed on a lateral flow device (LFD). This complex spreads by capillary action through the LFD. The test is read visually: if the result is positive, a red line (test line) appears in the result zone of the LFD membrane. The absence of a red line indicates a negative result. Whether or not gluten is present in the sample, a blue control line must also appear in the result zone for the test result to be valid. The absence of a blue control line indicates an invalid result.

Materials and Methods

Test Kit Information

- (a) *Kit name.*—AgraStrip Gluten G12 test kit.
- (b) *Cat. No.*—COKAL0200AS.
- (c) *Ordering information.*—Romer Labs, Technopark 1, 3430 Tulln, Austria; <http://www.romerlabs.com>.

Test Kit Components

- (a) *Tube of 10 AgraStrip Gluten G12 strips.*
- (b) *Dropper bottle (green label).*—Containing 35 mL extraction buffer.
- (c) *Dropper bottle (red label).*—Containing 35 mL dilution buffer.
- (d) *10 extraction tubes.*
- (e) *10 extraction tube caps.*
- (f) *10 extraction tube dropper tips.*
- (g) *10 dilution tubes with caps.*
- (h) *10 breakpoint swabs.*
- (i) *Tube holder.*

Apparatus

Recommended, not included in the kit:

- (a) *Mortar and pestle or grinder.*
- (b) *Balance.*

Precautions

- (a) The product must be stored in its original package,

between 15 and 25°C. Do not use components beyond the expiration date indicated on the kit labels. Do not open the product until needed.

- (b) Test strips must be kept inside their original packaging, closed as tightly as possible. Do not freeze.
- (c) Adhere to the instructions for test procedures.
- (d) Safety glasses, lab coats, and gloves should be worn as appropriate.

General Preparation

Once samples have been taken, the area where testing shall be performed must be clean and free of outside sources of gluten (especially airborne particulate) to avoid cross-contamination during the assay. Clean laboratory gloves are also recommended to avoid cross-contamination.

Sample Preparation

(a) *Ingredient/finished product testing.*—Obtain a representative sample (5 g recommended) and homogenize as appropriate to physical characteristics of sample (i.e., blend, crush, grind), then weigh 0.2 g of homogenized sample and add to extraction tube. Fill extraction tube to lower lip with extraction buffer, cap, and shake vigorously for 1 min. Remove cap and replace with dropper tip, then transfer three drops to a dilution tube. Fill dilution vial with dilution buffer to desired cutoff mark, cap, and shake for 15 s to mix. Remove cap and place a test strip vertically, with arrows pointing down, into the dilution tube. Allow liquid to travel up the strip to the flow level line (approximately 45 s), then remove strip and place upright in the tube holder. Allow strip to develop for 10 min before reading.

(b) *Swab testing.*—Fill extraction tube to lower lip with extraction buffer, then wet the end of a swab by dipping into the buffer. Wipe an area of 25 cm² using a cross-hatch pattern. Place the swab into the extraction tube, and carefully break off the tip at the prescored point. Cap the extraction tube and shake vigorously for 1 min. Remove cap and replace with dropper tip, then transfer three drops to a dilution tube. Fill dilution vial with dilution buffer to 5 ppm mark, cap, and shake for 15 s to mix. Remove cap and place a test strip vertically, with arrows pointing down, into the dilution tube. Allow liquid to travel up the strip to the flow level line (approximately 45 s), then remove strip and place upright in the tube holder. Allow strip to develop for 10 min before reading.

Interpretation of Results

- (a) One single blue line (control line) in the central result zone of the strip = negative result.
- (b) One red line (test line) and one blue line in the result zone = positive result. The sample contains gluten higher than the cutoff level and further investigations should be performed.
- (c) No control line appears = invalid result, regardless of whether the test line appears. In the case of an invalid result, please repeat the procedure with a new strip. If the problem persists, please contact Romer Labs before continuing further.

Validation Study

This validation study was conducted according to the AOAC Approved Final AgraStrip Gluten G12 Validation Outline v6.2 (February 3, 2014), prepared for Romer Labs by the AOAC Research Institute PTM program. Parameters tested include: cross-reactivity, interference, incurred samples, food matrixes, environmental surfaces, product consistency, stability, test kit variation, and robustness (8).

Preparation of Validation Materials

(a) Gluten-free food matrixes included Bob's Red Mill White Rice Flour, Ener-G Foods Gluten-Free Tapioca Loaf, Lucy's Gluten-Free Sugar Cookies, So Delicious Coconut Milk Vanilla Bean Ice Cream (package states gluten-free), and Chatfield's Double Dark Semi-Sweet Chocolate Chips (package states manufactured in a dedicated gluten-free facility).

(b) Reference materials used were gliadin produced by Prolamin Working Group (PWG), protein content = 91.4%, gliadin content = 88.2%, and wheat gluten produced by Sigma-Aldrich, Cat. No. G5004, Lot No. SLBD0196V, protein content = 84.0%, gliadin content as measured by AOAC *Official Methods of Analysis*SM (OMA) **2012.01** = 32.6% (Note: during extraction for OMA **2012.01**, it was observed the wheat gluten tended to agglutinate, as well as adhere to the sides of the extraction vial; therefore, it is unlikely all gliadin in the wheat gluten can be accurately measured in such a concentrated standard by this method).

Spiking of the test samples was performed using purified gliadin (88.2% gliadin by certificate of analysis) obtained from the PWG, or wheat gluten standard (WGS) obtained from Sigma-Aldrich (84.0% protein by certificate of analysis). PWG gliadin was dissolved at 1 mg/mL by weight in 60% ethanol (EtOH), which was calculated to be a working stock solution at 882 ppm gliadin. WGS was suspended at 1 mg/mL by weight in melted clarified cocoa butter, calculated to be a working stock suspension at 840 ppm gluten.

(c) Spiked samples were calculated using dilution factors to achieve the final concentrations indicated by AOAC Final AgraStrip Gluten G12 Validation Outline for each spike material and test matrix. Rice flour, bread, and cookies were spiked with gliadin, which was first dissolved to 1 mg/mL in 60% EtOH. This was further diluted into an amount of 60% EtOH calculated to saturate 1/10 of the final bulk matrix sample, then added to the small matrix portion, and EtOH was allowed to evaporate at room temperature. The small spiked portion was then ground by mortar and pestle to separate granules, and gradually mixed homogeneously by adding 1/10 final bulk volume unspiked matrix at a time, then remixing, until final bulk volume was achieved.

Ice cream, chocolate, and rice flour for stability, precision, repeatability, and robustness were spiked with Sigma-Aldrich WGS. Because gluten so readily binds to even small amounts of water, the gluten was suspended at 1 mg/mL in cocoa butter. Cocoa butter, when melted into a liquid fat, provided a water-free diluent, and method to incorporate the WGS into the matrix while retaining a coating of fat around the WGS granules to protect them from binding to water in the matrix. Cocoa butter originates from chocolate, and thus was appropriate for spiking into that matrix. Because cocoa butter has similar properties

Table 1. Cross-reactivity study results. Each food compound was tested ($n = 1$) for cross-reactivity with the AgraStrip Gluten G12 assay at the 20 ppm cutoff level, and screened for gliadin content using AOAC OMA 2012.01

Compound	Test line	Control line	20 ppm AgraStrip gluten threshold	AOAC OMA 2012.01 (ppm gliadin)
Almond flour	–	+	Negative	0.0
Amaranth flour	–	+	Negative	0.1
Arrowroot	–	+	Negative	0.0
Black bean flour	–	+	Negative	0.2
Brown rice flour	–	+	Negative	0.2
Buckwheat flour	–	+	Negative	–0.1
Chestnut flour	–	+	Negative	0.0
Coconut flour	–	+	Negative	0.8
Coffee	–	+	Negative	0.0
Corn meal	–	+	Negative	0.3
Dried fruits	–	+	Negative	0.0
Egg powder	–	+	Negative	0.0
Fava bean flour	–	+	Negative	0.4
Flax seed meal	–	+	Negative	0.6
Garfava flour	–	+	Negative	0.1
Green pea flour	–	+	Negative	0.0
Guar gum	–	+	Negative	0.6
Hazelnut flour	–	+	Negative	0.0
Lentil flour	–	+	Negative	0.1
Lima bean flour	–	+	Negative	0.9
Meats	–	+	Negative	0.0
Milk powder	–	+	Negative	0.4
Millet flour	–	+	Negative	0.0
Oat flour	–	+	Negative	0.2
Potato starch	–	+	Negative	0.0
Quinoa flour	–	+	Negative	0.2
Romano bean flour	–	+	Negative	0.0
Sesame flour	–	+	Negative	0.1
Sorghum flour	–	+	Negative	1.6
Soya flour	–	+	Negative	8.1
Spices	–	+	Negative	0.0
Sweet rice flour	–	+	Negative	0.4
Tapioca flour	–	+	Negative	0.1
Tea	–	+	Negative	0.9
White bean flour	–	+	Negative	0.8
White rice flour	–	+	Negative	0.0
Xanthan gum	–	+	Negative	0.0
Yellow pea flour	–	+	Negative	0.1

Table 2. Interference study results. Each food compound was spiked with 5 ppm gliadin (approximately 10 ppm gluten), then tested for interference with the AgraStrip Gluten G12 assay at the 5 ppm and 20 ppm cutoff levels ($n = 1$)

Compound	5 ppm AgraStrip gluten threshold			20 ppm AgraStrip gluten threshold		
	Test line	Control line	Result	Test line	Control line	Result
Almond flour	+	+	Positive	-	+	Negative
Amaranth flour	+	+	Positive	-	+	Negative
Arrowroot	+	+	Positive	-	+	Negative
Black bean flour	+	+	Positive	-	+	Negative
Brown rice flour	+	+	Positive	-	+	Negative
Buckwheat flour	+	+	Positive	-	+	Negative
Chestnut flour	+	+	Positive	-	+	Negative
Coconut flour	+	+	Positive	-	+	Negative
Coffee	+	+	Positive	-	+	Negative
Corn meal	+	+	Positive	-	+	Negative
Dried fruits	+	+	Positive	-	+	Negative
Egg powder	+	+	Positive	-	+	Negative
Fava bean flour	+	+	Positive	-	+	Negative
Flax seed meal	+	+	Positive	-	+	Negative
Garfava flour	+	+	Positive	-	+	Negative
Green pea flour	+	+	Positive	-	+	Negative
Guar gum	+	+	Positive	-	+	Negative
Hazelnut flour	+	+	Positive	-	+	Negative
Lentil flour	+	+	Positive	-	+	Negative
Lima bean flour	+	+	Positive	-	+	Negative
Meats	+	+	Positive	-	+	Negative
Milk powder	+	+	Positive	-	+	Negative
Millet flour	+	+	Positive	-	+	Negative
Oat flour	+	+	Positive	-	+	Negative
Potato starch	+	+	Positive	-	+	Negative
Quinoa flour	+	+	Positive	-	+	Negative
Romano bean flour	+	+	Positive	-	+	Negative
Sesame flour	+	+	Positive	-	+	Negative
Sorghum flour	+	+	Positive	-	+	Negative
Soya flour	+	+	Positive	-	+	Negative
Spices	+	+	Positive	-	+	Negative
Sweet rice flour	+	+	Positive	-	+	Negative
Tapioca flour	+	+	Positive	-	+	Negative
Tea	+	+	Positive	-	+	Negative
White bean flour	+	+	Positive	-	+	Negative
White rice flour	+	+	Positive	-	+	Negative
Xanthan gum	+	+	Positive	-	+	Negative
Yellow pea flour	+	+	Positive	-	+	Negative

Table 3. Independent laboratory incurred sample study results

Matrix	Gliadin (gluten) spike concentration	AgraStrip detection threshold (ppm gluten)	N ^a	Candidate			Avg. AOAC OMA 2012.01 results (ppm gliadin) <i>n</i> = 3	
				x ^b	POD _C ^c	95% CI ^d		
Incurred bread	0 ppm	5	30	0	0.00	0.00, 0.11	<2.5	
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
	14.97 ppm (29.94)	5	30	30	1.00	0.89, 1.00		14.97
		10	30	30	1.00	0.89, 1.00		
		20	30	0	0.00	0.00, 0.11		

^a N = Number of test portions.

^b x = Number of positive test portions.

^c POD_C = Candidate method confirmed positive outcomes divided by the total number of trials.

^d 95% Confidence intervals.

to coconut oil, being a solid at room temperature, it was also deemed appropriate as a spike diluent for ice cream. From the 1 mg/mL WGS stock, a 1:10 dilution was made before spiking a calculated amount into 1/10 the final bulk volume of melted ice cream or chocolate. Additional melted ice cream or chocolate was added 1/10 the final bulk volume at a time, then remixed, until the final bulk volume was achieved. The chocolate was then frozen to -20°C and ground. Rice flour was spiked in a similar method, while kept warm to keep the cocoa butter from setting fully, then the final bulk volume allowed to cool to room temperature and ground once more.

All spike methods were tested prior to performing the validation study, and homogeneity testing of five samples from each spiked matrix demonstrated a homogeneously distributed spike. This testing, however, did not take into account the amount of time required to aliquot the full number of samples needed for the study, during which the spike in ice cream gradually was exposed to enough water in the matrix that it began to agglutinate as well as adhere to tube and container walls, pipet tips, etc. This homogeneity testing, while taken from representative areas around the bulk sample, could not necessarily detect small “hot spots” of concentrated spike material within the bulk.

(d) The incurred matrix study, performed by the independent laboratory, involved a spiking strategy similar to that of Romer Labs, although the entire gluten free bread mix matrix was saturated with a more dilute solution of gliadin in 60% EtOH, and allowed to dry 18 h before baking. Thirty ppm gliadin was spiked into the bread mix prior to baking in order to achieve a final 15 ppm gliadin concentration (30 ppm gluten) in the baked bread, per AOAC Final AgraStrip Gluten G12 Validation Outline V6.2, Tables 2 and 5 (8). The bread mix was prepared and baked following the manufacturer’s instructions, cooled for 1 h, then dried 18 h using a LabConco freeze-dry system and homogenized by mortar and pestle.

(e) For environmental surface testing, a 25 cm² area of a stainless steel coupon was spiked with the 1 mg/mL stock solution of gliadin in 60% EtOH by pipetting a volume onto the surface calculated to spike to the concentrations indicated in AOAC Final AgraStrip Gluten G12 Validation Outline

V6.2. The coupon was then covered and incubated at room temperature until the EtOH fully evaporated before swabbing.

Methodology

Analyst 1 spiked sample matrixes in bulk, aliquotted to tubes and extracted, then blind-coded and randomized extracts. Analyst 2 then ran and read the blinded extracts, after which results were decoded and analyzed. Where indicated in the protocol, a third analyst tested cross-reactivity and food matrix samples using reference method AOAC OMA 2012.01. *Note:* Previous studies have documented that results of AOAC OMA 2012.01 have a %RSD ranging from 22 to 52% depending on gliadin concentration in the sample (9).

Results

Cross-Reactivity Study

Thirty-eight gluten-free labeled food compounds were screened, unspiked, against the AgraStrip Gluten G12 assay at the 20 ppm threshold for potential cross-reactivity as well as for quantitative gliadin content using AOAC OMA 2012.01 (LOD = 2.5 ppm). The results, shown in Table 1, indicate no cross-reactivity with any of the screened compounds. The authors note that soya flour, at 8.1 ppm gliadin, had a higher level of contamination than desired for this study. Four different

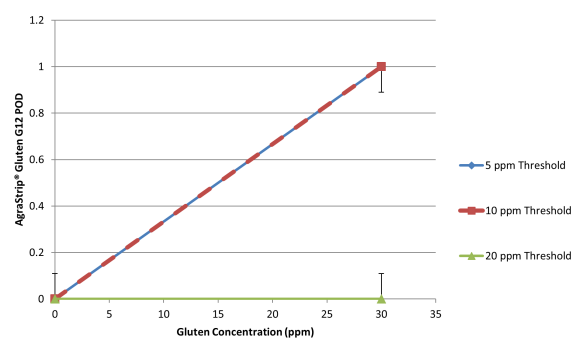


Figure 1. AgraStrip Gluten G12 POD versus calculated gluten concentration of gliadin spike for incurred bread.

Table 4. Independent laboratory food matrix testing results for gliadin-spiked rice flour

Matrix	Gliadin (gluten) spike concentration	AgraStrip detection threshold (ppm gluten)	N ^a	Candidate			Avg. AOAC OMA 2012.01 results (ppm gliadin) n = 3
				x ^b	POD _C ^c	95% CI ^d	
Rice flour	0 ppm	5	30	0	0.00	0.00, 0.11	<2.5
		10	30	0	0.00	0.00, 0.11	
		20	30	0	0.00	0.00, 0.11	
	3 ppm (6)	5	30	0	0.00	0.00, 0.11	2.92
		10	30	0	0.00	0.00, 0.11	
		20	30	0	0.00	0.00, 0.11	
	8 ppm (16)	5	30	30	1.00	0.89, 1.00	7.54
		10	30	3	0.10	0.03, 0.26	
		20	30	0	0.00	0.00, 0.11	
	15 ppm (30)	5	30	30	1.00	0.89, 1.00	15.18
		10	30	30	1.00	0.89, 1.00	
		20	30	3	0.10	0.03, 0.26	
25 ppm (50)	5	30	30	1.00	0.89, 1.00	23.03	
	10	30	30	1.00	0.89, 1.00		
	20	30	30	1.00	0.89, 1.00		

^a N = Number of test portions.

^b x = Number of positive test portions.

^c POD_C = Candidate method confirmed positive outcomes divided by the total number of trials.

^d 95% Confidence intervals.

brands of soya flour were tested by AOAC OMA 2012.01, all having similar or higher levels of contamination, and the brand screened against AgraStrip Gluten G12 represents the brand with the lowest level of contamination.

Interference Study

Each of the 38 compounds screened in the cross-reactivity study was spiked with 5 ppm gliadin and assayed with AgraStrip Gluten G12 at the 5 ppm and 20 ppm threshold levels. At 5 ppm, all spiked compounds returned a positive result, and at 20 ppm all compounds were negative, as shown in Table 2. The authors noted that soya flour, which with the 8.1 ppm contamination level plus the 5 ppm gliadin spike was calculated to have a gliadin concentration of 13.1 ppm, had a noticeably brighter test line at the 5 ppm threshold than the other compounds screened, but was still negative at the 20 ppm threshold.

Incurred Samples Study

The independent laboratory prepared “Bob’s Red Mill Homemade Wonderful” gluten-free bread mix in unspiked and 30 ppm (target 15 ppm after baking) gliadin spike portions. The bread mix ingredients are as follows: stone ground garbanzo bean flour, potato starch, corn starch, sweet white sorghum flour, tapioca flour, evaporated cane juice, fava bean flour, xanthan gum, active dry yeast, potato flour, sea salt (magnesium carbonate as flowing agent), guar gum, and soy lecithin. The yeast package was bloomed in warm milk for 5 min, then added to bread mix, along with one egg, ¼ cup melted butter, and

1 tsp cider vinegar. The ingredients were mixed, placed into a baking pan, then covered and allowed to rise for 30 min. The bread was next baked in a Hot Point model R3787W oven at 375° F (approximately 190°C) for 50 min, and then allowed to cool on the bench for 1 h. The cooled bread was freeze-dried, homogenized, and tested with AgraStrip Gluten G12. Table 3 and Figure 1 show that unspiked samples all returned a negative result. For the spiked samples, at 30 ppm gluten, the 5 and 10 ppm thresholds returned all positive results, while the 20 ppm threshold returned all negative results.

Food Matrix Testing

The following food matrixes were pre-screened by AOAC OMA 2012.01 for gliadin content: white rice = 0.0 ppm, gluten-free cookies = 0.18 ppm, gluten-free bread = 0.08 ppm,

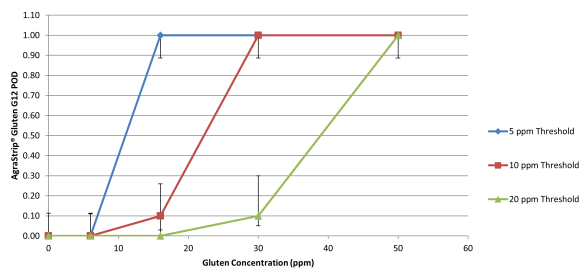


Figure 2. AgraStrip Gluten G12 POD versus calculated gluten concentration for gliadin spike into rice flour by independent laboratory.

Table 5. Food matrix testing results for gliadin-spiked rice flour

Matrix	Gliadin (gluten) spike concentration	AgraStrip detection threshold (ppm gluten)	N ^a	Candidate			Avg. AOAC OMA 2012.01 results (ppm gliadin) <i>n</i> = 3	
				x ^b	POD _C ^c	95% CI ^d		
Rice flour	0 ppm	5	30	0	0.00	0.00, 0.11	<2.5	
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
	3 ppm (6)	5	30	12	0.40	0.25, 0.58		3.55
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
	8 ppm (16)	5	30	30	1.00	0.89, 1.00		4.98
		10	30	13	0.43	0.27, 0.61		
		20	30	3	0.10	0.03, 0.26		
	15 ppm (30)	5	30	30	1.00	0.89, 1.00		13.47
		10	30	30	1.00	0.89, 1.00		
		20	30	30	1.00	0.89, 1.00		
	25 ppm (50)	5	30	30	1.00	0.89, 1.00		23.50
		10	30	30	1.00	0.89, 1.00		
		20	30	30	1.00	0.89, 1.00		

^a N = Number of test portions.

^b x = Number of positive test portions.

^c POD_C = Candidate method confirmed positive outcomes divided by the total number of trials.

^d 95% Confidence intervals.

gluten-free ice cream = 0.15 ppm, gluten-free chocolate = 0.05 ppm. For each of the 5, 10, and 20 ppm threshold levels, the aforementioned food matrixes (*n* = 30) were spiked with 0, 3, 8, 15, and 25 ppm gliadin (rice flour, cookies, bread) or WGS (ice cream, dark chocolate), then assayed with AgraStrip Gluten G12. As well, three replicates of each spike level were screened for gliadin content using AOAC OMA 2012.01. The independent laboratory also performed a food matrix test using rice flour.

For rice flour tested by the independent laboratory, shown in Table 4 and Figure 2, the 0, 3, 8, 15, and 25 ppm gliadin spikes returned gliadin concentrations at <2.5, 2.92, 7.54, 15.18, and 23.03 ppm, respectively. At 0 and 3 ppm gliadin spikes, AgraStrip Gluten G12 had POD of 0.00 at all three gluten thresholds. At 8 ppm gliadin spike, POD was 1.00, 0.10, and 0.00 at the 5, 10, and 20 ppm gluten thresholds, respectively.

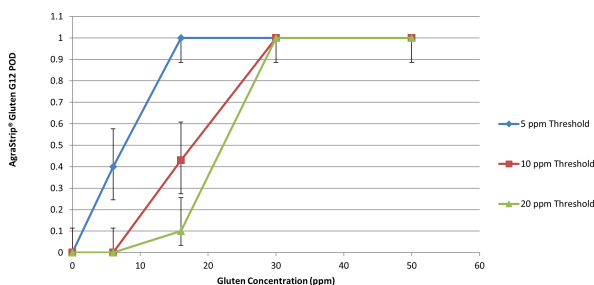


Figure 3. AgraStrip Gluten G12 POD versus calculated gluten concentration for gliadin spike into rice flour.

At 15 ppm gliadin spike, PODs were 1.00, 1.00, and 0.10 at the 5, 10, and 20 ppm gluten thresholds, respectively. At 25 ppm gliadin spike, POD was 1.00 at all three thresholds.

For rice flour, shown in Table 5 and Figure 3, the 0, 3, 8, 15, and 25 ppm gliadin spikes returned gliadin concentrations at <2.5, 3.55, 4.98, 13.47, and 23.50 ppm, respectively. At 0 ppm gliadin (0 ppm gluten) spike, AgraStrip Gluten G12 had a POD of 0.00 at all three gluten thresholds. At 3 ppm gliadin (6 ppm gluten), PODs were 0.40, 0.00, and 0.00 at the 5, 10, and 20 ppm thresholds, respectively. At 8 ppm gliadin (16 ppm gluten) spike, there were PODs of 1.00, 0.43, and 0.10 at the 5, 10, and 20 ppm gluten thresholds, respectively. At 15 and 25 ppm gliadin (30 and 50 ppm gluten) spikes, POD was 1.00 at all three thresholds. Figure 4 plots difference in probability of detection values (dPOD) for rice flour between the sponsor and independent laboratories for gliadin-spiked rice flour.

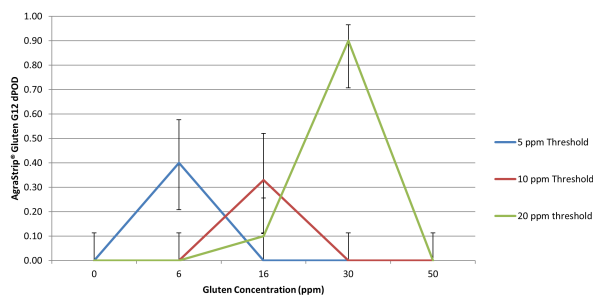


Figure 4. dPOD of AgraStrip Gluten G12 POD results between sponsor and independent laboratories for gliadin-spiked rice flour.

Table 6. Food matrix testing results for gliadin-spiked gluten-free cookies

Matrix	Gliadin (gluten) spike concentration	AgraStrip detection threshold (ppm gluten)	N ^a	Candidate			Avg. AOAC OMA 2012.01 results (ppm gliadin) <i>n</i> = 3
				x ^b	POD _C ^c	95% CI ^d	
Cookies	0 ppm	5	30	0	0.00	0.00, 0.11	0.30
		10	30	0	0.00	0.00, 0.11	
		20	30	0	0.00	0.00, 0.11	
	3 ppm (6)	5	30	28	0.93	0.79, 0.98	2.65
		10	30	26	0.87	0.70, 0.95	
		20	30	0	0.00	0.00, 0.11	
	8 ppm (16)	5	30	30	1.00	0.89, 1.00	8.28
		10	30	30	1.00	0.89, 1.00	
		20	30	4	0.13	0.05, 0.30	
	15 ppm (30)	5	30	30	1.00	0.89, 1.00	11.67
		10	30	30	1.00	0.89, 1.00	
		20	30	29	0.97	0.83, 1.00	
25 ppm (50)	5	30	30	1.00	0.89, 1.00	17.75	
	10	30	30	1.00	0.89, 1.00		
	20	30	30	1.00	0.89, 1.00		

^a N = Number of test portions.

^b x = Number of positive test portions.

^c POD_C = Candidate method confirmed positive outcomes divided by the total number of trials.

^d 95% Confidence intervals.

and independent laboratories, demonstrating a difference of 0.4 at 6 ppm gluten spike within the 5 ppm threshold, 0.52 at 16 ppm gluten spike within the 10 ppm threshold, and within the 20 ppm threshold 0.26 at 16 ppm and 0.97 at 30 ppm gluten spikes. This is likely due to differences in spiking technique, as well as differences in operator interpretation of test lines when allergen concentrations are at levels close to the LOD.

For gluten-free cookies, shown in Table 6 and Figure 5, the 0, 3, 8, 15, and 25 ppm gliadin spikes returned gliadin concentrations at 0.30, 2.65, 8.28, 11.67, and 17.75 ppm, respectively. At 0 ppm gliadin (0 ppm gluten) spike, AgraStrip Gluten G12 had a POD of 0.00 at all three gluten thresholds. At 3 ppm gliadin (6 ppm gluten), PODs were 0.93, 0.87, and 0.00 at the 5, 10, and 20 ppm thresholds, respectively. At 8 ppm gliadin (16 ppm gluten) spike, there were PODs of 1.00, 1.00, and 0.13 at the 5, 10, and 20 ppm gluten thresholds, respectively. At 15 ppm gliadin (30 ppm gluten) spike, there were PODs of 1.00, 1.00, and 0.97 at the 5, 10, and 20 ppm thresholds, respectively. At 25 ppm gliadin (50 ppm gluten) spike, POD was 1.00 at all three thresholds.

For gluten-free bread, shown in Table 7 and Figure 6, the 0, 3, 8, 15, and 25 ppm gliadin spikes returned gliadin concentrations at <2.5, 2.32, 7.60, 13.37, and 18.75 ppm, respectively. At 0 ppm gliadin (0 ppm gluten) spike, AgraStrip Gluten G12 had a POD of 0.00 at all three gluten thresholds. At 3 ppm gliadin (6 ppm gluten), PODs were 1.00, 0.43, and 0.00 at the 5, 10, and 20 ppm thresholds, respectively. At 8 ppm gliadin (16 ppm gluten) spike, there were PODs of 1.00, 0.93, and 0.10 at the 5, 10, and 20 ppm gluten thresholds, respectively. At the 15 ppm gliadin (30 ppm gluten) spike there were PODs of 1.00, 1.00, and 0.97 at the 5, 10, and 20 ppm thresholds, respectively. At

the 25 ppm gliadin (50 ppm gluten) spike, POD was 1.00 at all three thresholds.

For gluten-free ice cream, shown in Table 8 and Figure 7, the 0, 3, 8, 15, and 25 ppm WGS spikes returned gliadin concentrations at <2.5, 6.18, 21.42, 38.17, and 30.43 ppm, respectively. At 0 ppm WGS spike, AgraStrip Gluten G12 had a POD of 0.00 at all three gluten thresholds. At 3 ppm WGS, PODs were 1.00, 0.93, and 0.00 at the 5, 10, and 20 ppm thresholds, respectively. At 8 ppm WGS spike, there were PODs of 1.00, 1.00, and 0.33 at the 5, 10, and 20 ppm gluten thresholds, respectively. At 15 and 25 ppm WGS spike, POD was 1.00 at all three thresholds.

For gluten-free chocolate, shown in Table 9 and Figure 8, the 0, 3, 8, 15, and 25 ppm WGS spikes returned gliadin concentrations at <2.5, 4.36, 5.61, 13.15, and 32.87 ppm, respectively. At 0 ppm WGS spike, AgraStrip Gluten G12 had a POD of 0.00 at all three

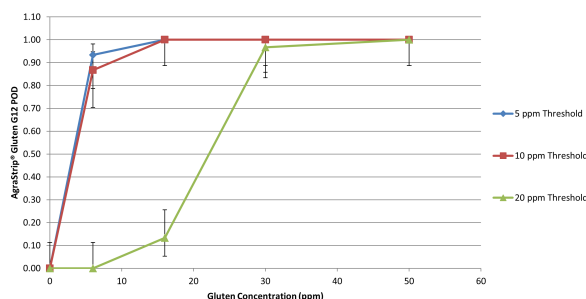


Figure 5. AgraStrip Gluten G12 POD versus calculated gluten concentration for gliadin spike into gluten-free cookies.

Table 7. Food matrix testing results for gliadin-spiked gluten-free bread

Matrix	Gliadin (gluten) spike concentration	AgraStrip detection threshold (ppm gluten)	N ^a	Candidate			Avg. AOAC OMA 2012.01 results (ppm gliadin) <i>n</i> = 3
				x ^b	POD _C ^c	95% CI ^d	
Bread	0 ppm	5	30	0	0.00	0.00, 0.11	<2.5
		10	30	0	0.00	0.00, 0.11	
		20	30	0	0.00	0.00, 0.11	
	3 ppm (6)	5	30	30	1.00	0.89, 1.00	2.32
		10	30	13	0.43	0.27, 0.61	
		20	30	0	0.00	0.00, 0.11	
	8 ppm (16)	5	30	30	1.00	0.89, 1.00	7.60
		10	30	28	0.93	0.79, 0.98	
		20	30	3	0.10	0.03, 0.26	
	15 ppm (30)	5	30	30	1.00	0.89, 1.00	13.37
		10	30	30	1.00	0.89, 1.00	
		20	30	29	0.97	0.83, 1.00	
	25 ppm (50)	5	30	30	1.00	0.89, 1.00	18.75
		10	30	30	1.00	0.89, 1.00	
		20	30	30	1.00	0.89, 1.00	

^a N = Number of test portions.

^b x = Number of positive test portions.

^c POD_C = Candidate method confirmed positive outcomes divided by the total number of trials.

^d 95% Confidence intervals.

gluten thresholds. At 3 ppm WGS, PODs were 0.10, 0.00, and 0.00 at the 5, 10, and 20 ppm thresholds, respectively. At 8 ppm WGS spike, there were PODs of 0.93, 0.23, and 0.03 at the 5, 10, and 20 ppm gluten thresholds, respectively. At 15 ppm WGS spike there were PODs of 1.00, 1.00, and 0.57 at the 5, 10, and 20 ppm thresholds, respectively. At 25 ppm WGS spike, POD was 1.00 at all three thresholds.

Rice flour spiked with WGS at 10000 ppm was also tested to determine whether the assay could experience hook effect at high contamination levels. Results, shown in Table 10, indicate positive results at all three thresholds.

Environmental Surface Testing

Both the independent laboratory and the authors prepared 25 cm² stainless steel coupons as unspiked with *n* = 5, fractional recovery spike of 3.5 μg gliadin (7 μg gluten) with *n* = 30, and high level spike at 17.7 μg gliadin (35.4 μg gluten) with *n* = 5. Both labs recorded AgraStrip Gluten G12 results as all negative for the unspiked coupons, and all positive for the high level, as shown in Tables 11 and 12, and Figures 9 and 10. For fractional recovery, the independent lab recorded 19/30 positive, and the authors recorded 18/30 positive. Figure 11 plots dPOD of environmental surface testing results between sponsor and independent laboratories. There is no significant difference

between the internal validation data and the independent laboratory data at any spike level for the environmental surface.

Test Kit Variation Study

Result variation was assessed between six different test kits of the same production lot using unspiked and 15 ppm WGS-spiked rice flour samples (*n* = 10 at the 10 and 20 ppm thresholds). Results between the six test kits did not demonstrate variability, showing negative results in all unspiked samples. At the 15 ppm WGS spike, all 10 ppm threshold results were POD of 1.00, while

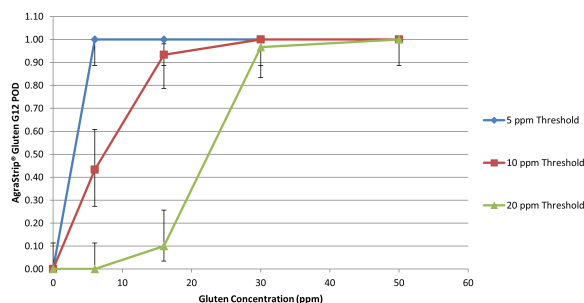


Figure 6. AgraStrip Gluten G12 POD versus calculated gluten concentration for gliadin spike into gluten-free bread.

Table 8. Food matrix testing results for WGS-spiked gluten-free ice cream

Matrix	WGS spike gluten concentration	AgraStrip detection threshold (ppm gluten)	N ^a	Candidate			Avg. AOAC OMA 2012.01 Results (ppm Gliadin) <i>n</i> = 3
				x ^b	POD _C ^c	95% CI ^d	
Ice cream	0 ppm	5	30	0	0.00	0.00, 0.11	<2.5
		10	30	0	0.00	0.00, 0.11	
		20	30	0	0.00	0.00, 0.11	
	3 ppm	5	30	30	1.00	0.89, 1.00	6.18
		10	30	28	0.93	0.79, 0.98	
		20	30	0	0.00	0.00, 0.11	
	8 ppm	5	30	30	1.00	0.89, 1.00	21.42
		10	30	30	1.00	0.89, 1.00	
		20	30	10	0.33	0.19, 0.51	
	15 ppm	5	30	30	1.00	0.89, 1.00	38.17
		10	30	30	1.00	0.89, 1.00	
		20	30	30	1.00	0.89, 1.00	
25 ppm	5	30	30	1.00	0.89, 1.00	30.43	
	10	30	30	1.00	0.89, 1.00		
	20	30	30	1.00	0.89, 1.00		

^a N = Number of test portions.

^b x = Number of positive test portions.

^c POD_C = Candidate method confirmed positive outcomes divided by the total number of trials.

^d 95% Confidence intervals.

all 20 ppm threshold results were POD of 0.00 (*see* Table S1 in the supplemental data).

Product Consistency Study

Product consistency testing was performed between three production lots of AgraStrip Gluten G12 test kits using unspiked and 15 ppm WGS-spiked rice flour samples (*n* = 10 at 10 and 20 ppm thresholds). As shown in Table S2 in the supplemental data, results are consistent among the three production lots, showing negative results in all unspiked samples. At the 15 ppm WGS spike, all 10 ppm threshold results were POD of 1.00, while all 20 ppm threshold results were POD of 0.00 (*see* Table S2 in the supplemental data).

Stability Study

Accelerated stability testing was performed in a similar manner, but with the test kits incubated at 42°C and assessed at 0, 10, 20, 35, 50, and 90 days (*see* Table S3 in the supplemental data). Excepting Day 50, all other results showed a POD of 0.00 in unspiked samples; 15 ppm WGS spiked samples showed a POD of 1.00 in all 10 ppm threshold strips tested, and all 20 ppm thresholds were a POD of 0.00. In the case of Day 50, the 20 ppm threshold showed a POD of 0.40 in the 20 ppm threshold, which may have been due to variation in spike homogeneity because there was no anomaly in POD of Day 90. Stability of AgraStrip Gluten G12 test kits was assessed at 0, 3, 9, and 12 months using unspiked and 15 ppm WGS-spiked rice flour samples (*n* = 10 at 10 and 20 ppm thresholds), with

a 15 month time point to be tested in the future (*see* Table S4 in the supplemental data). Results show a POD of 0.00 in all unspiked samples, while the 15 ppm WGS spike showed POD of 1.00 at all 10 ppm threshold strips tested and 0.00 at all 20 ppm thresholds tested.

Robustness Study

Robustness of the AgraStrip Gluten G12 assay was assessed by altering several test parameters: extraction time, extraction buffer level, and AgraStrip incubation time. Unspiked and 15 ppm WGS-spiked rice flour samples were tested at the 10 and 20 ppm thresholds. Results, shown in Table S5 in the supplemental data, indicate that 0 min extraction time produced negative results, with some positive recovery when AgraStrips

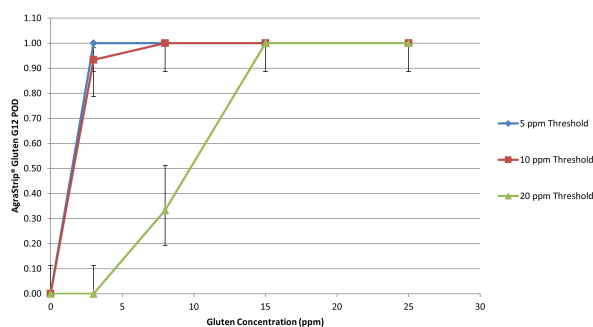


Figure 7. AgraStrip Gluten G12 POD versus gluten concentration of WGS spike into gluten-free ice cream.

Table 9. Food matrix testing results for WGS-spiked gluten-free chocolate

Matrix	WGS spike gluten concentration	AgraStrip detection threshold (ppm gluten)	N ^a	Candidate			Avg. AOAC OMA 2012.01 results (ppm gliadin) <i>n</i> = 3	
				x ^b	POD _C ^c	95% CI ^d		
Chocolate	0 ppm	5	30	0	0.00	0.00, 0.11	<2.5	
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
	3 ppm	5	30	3	0.10	0.03, 0.26		4.36
		10	30	0	0.00	0.00, 0.11		
		20	30	0	0.00	0.00, 0.11		
	8 ppm	5	30	28	0.93	0.79, 0.98		5.61
		10	30	7	0.23	0.12, 0.41		
		20	30	1	0.03	0.00, 0.17		
	15 ppm	5	30	30	1.00	0.89, 1.00		13.15
		10	30	30	1.00	0.89, 1.00		
		20	30	17	0.57	0.39, 0.73		
25 ppm	5	30	30	1.00	0.89, 1.00	32.87		
	10	30	30	1.00	0.89, 1.00			
	20	30	30	1.00	0.89, 1.00			

^a N = Number of test portions.

^b x = Number of positive test portions.

^c POD_C = Candidate method confirmed positive outcomes divided by the total number of trials.

^d 95% Confidence intervals.

were incubated for 15 min. Half-full extraction buffer produced all positive results at 15 ppm in both 10 and 20 ppm thresholds. Five min extractions, with over-full buffer, produced positive results at 15 ppm spike in the 10 ppm threshold, and negative in the 20 ppm threshold.

Discussion

The results of this study demonstrate that the AgraStrip Gluten G12 test kit will neither cross-react with a broad spectrum of gluten-free food compounds, nor will those compounds interfere with a positive result. Environmental surface spikes were recovered as expected, demonstrating no false-positive results, fractional recovery just below the LOD, and recovery at high spike concentration. The assay demonstrated consistent results between different production lots, as well as between different kits within the same lot. Ongoing stability studies show that the AgraStrip Gluten G12 kit is stable over a period of 3 months, as well as over a period of 50 days during accelerated stability at 42°C. Results of the robustness study indicated a 0 min protein extraction is not advisable, nor is halving the amount of extraction buffer used, as this effectively doubles the gluten concentration in the extract. A 5 min extraction, over-filling the extraction buffer, and varying the AgraStrip incubation time did not significantly affect the test outcome.

Food matrix testing indicates the AgraStrip Gluten G12 assay is capable of detecting the presence of 5, 10, and 20 ppm gliadin spike levels in gluten-free rice flour, cookies, and bread at the respective 5, 10, and 20 ppm assay thresholds. Per the validation outline, gliadin was spiked at 0, 3, 8, 15,

and 25 ppm levels, though it comprises only approximately 50% of the total protein content of gluten. The AgraStrip Gluten G12 antibodies detect the presence of gluten through a gliadin epitope; however, the assay is designed to reflect gluten content (while AOAC OMA **2012.01** measures gliadin content). Therefore, food matrix testing where gliadin was spiked at 3, 8, 15, and 25 ppm represents gluten concentrations of approximately 6, 16, 30, and 50 ppm in the AgraStrip Gluten G12 assay. During incurred matrix testing, no spike recovery at the 20 ppm threshold was observed, despite the sample having measured at approximately 15 ppm gliadin, or 30 ppm gluten. Because AOAC OMA **2012.01** has a %RSD ranging from 22 to 52% (9), the range for the amount of gliadin in the baked bread allows for the possibility that the amount of gliadin in the baked bread could have been below the 20 ppm threshold.

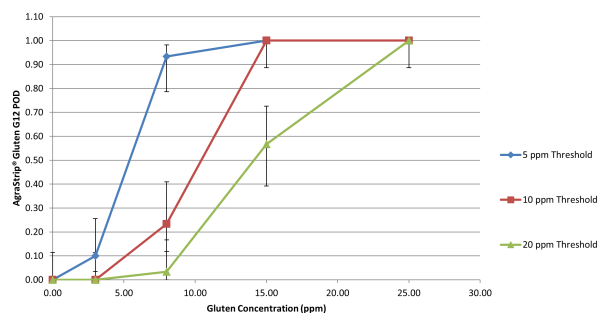


Figure 8. AgraStrip Gluten G12 POD versus gluten concentration of WGS spike into gluten-free chocolate.

Table 10. Food matrix testing results for hook effect at high WGS spike concentration in rice flour

Matrix	WGS spike gluten concentration	AgraStrip detection threshold (ppm gluten)	N ^a	Candidate			Avg. AOAC OMA 2012.01 results (ppm gliadin) <i>n</i> = 3
				x ^b	POD _C ^c	95% CI ^d	
Rice flour hook effect	10,000 ppm	5	10	10	1.00	0.72, 1.00	6,640
		10	10	10	1.00	0.72, 1.00	
		20	10	10	1.00	0.72, 1.00	

^a N = Number of test portions.

^b x = Number of positive test portions.

^c POD_C = Candidate method confirmed positive outcomes divided by the total number of trials.

^d 95% Confidence intervals.

Table 11. Independent laboratory environmental surface test results.

Matrix	Gliadin spike concentration, µg	N ^a	Presumptive		
			x ^b	POD _C ^c	95% CI ^d
Stainless steel 25 cm ²	0	5	0	0.00	0.00, 0.43
	3.5	30	19	0.63	0.46, 0.78
	17.6	5	5	1.00	0.57, 1.00

^a N = Number of test portions.

^b x = Number of positive test portions.

^c POD_C = Candidate method confirmed positive outcomes divided by the total number of trials.

^d 95% Confidence intervals.

Table 12. Environmental surface test results

Matrix	Gliadin spike concentration, µg	N ^a	Presumptive		
			x ^b	POD _C ^c	95% CI ^d
Stainless steel 25 cm ²	0	5	0	0.00	0.00, 0.43
	3.5	30	18	0.60	0.42, 0.75
	17.7	5	5	1.00	0.57, 1.00

^a N = Number of test portions.

^b x = Number of positive test portions.

^c POD_C = Candidate method confirmed positive outcomes divided by the total number of trials.

^d 95% Confidence intervals.

The AgraStrip Gluten G12 assay has been observed to perform reliably when testing heat-treated samples, due to the highly stable sequential epitope which the G12 antibody detects. The high gliadin concentrations observed during the gluten-free ice cream testing reflect the difficulty of spiking complete WGS into a food matrix containing water. The gluten particles drew water in, bloomed, and tended to adhere to tube walls and pipet tips, as well as agglutinate. This agglutinative effect caused by the water present in ice cream likely produced heterogeneity of gluten concentrations observed with that matrix. A gliadin spike into the ice cream likely would have produced better homogeneity. Because a fat, cocoa butter, was used to suspend and dilute the WGS for chocolate, and chocolate had far less water content, better homogeneity was achieved, and the results more accurately reflect detection of the spike levels of WGS, despite the high levels of tannins present in 70% cacao dark chocolate tested. Still, at the 8 ppm threshold in chocolate two

false-negative results were observed. These results were due to the chocolate hardening in the bottom of the extraction tube before it could be fully mixed with the extraction buffer. These results do not indicate a failure of the assay to detect gluten, but rather reflect the challenge of spiking and manipulating, within such a large study, a matrix having chocolate's physical properties. Over the entire study no false-positive results were observed in any blank sample.

Conclusions

The AgraStrip Gluten G12 test kit is a consistent, stable, robust, and accurate lateral flow assay for the qualitative detection of gluten in raw ingredients, processed food and finished food products, and environmental surfaces.

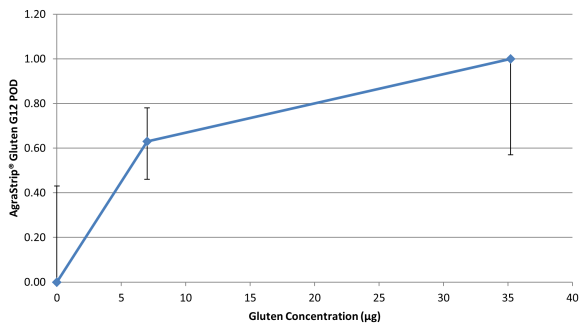


Figure 9. AgraStrip Gluten G12 POD versus calculated gluten concentration of gliadin-spiked environmental surface by independent laboratory.

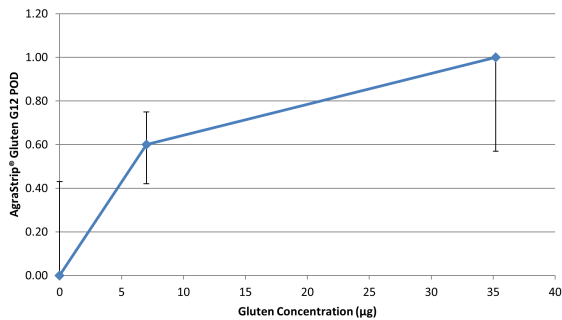


Figure 10. AgraStrip Gluten G12 POD versus calculated gluten concentration of gliadin-spiked environmental surface.

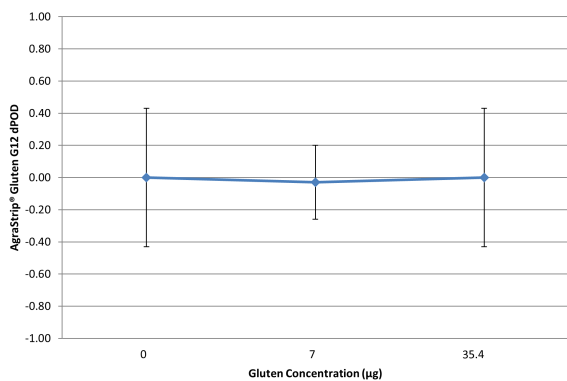


Figure 11. dPOD of AgraStrip Gluten G12 POD results between sponsor and independent laboratories for gliadin-spiked environmental surface.

Acknowledgments

The authors wish to thank Romer Labs, Inc. for funding, in whole, this validation study. The authors acknowledge Erin Crowley, M. Joseph Benzinger Jr., Patrick Bird, Benjamin Bastin, Jonathan Flannery, Kateland Koch, Paige Bedinghaus, William Judd, James Agin, and David Goins of Q Laboratories, Inc., Cincinnati, OH, for their participation in independent laboratory evaluations.

References

- (1) Moron, B., Cebolla, A., Manyani, H., Alvarez-Maqueda, M., Megias, M., Thomas, M., Lopez, M.C., & Sousa, C. (2008) *Am. J. Clin. Nutr.* **87**, 405–414. <http://ajcn.nutrition.org/content/87/2/405.long>
- (2) Moron, B., Bethune, M.T., Comino, I., Manyani, H., Ferragud, M., Lopez, M.C., Cebolla, A., Khosla, C., & Sousa, C. (2008) *PLoS One* **3**, e2294. <http://dx.doi.org/10.1371/journal.pone.0002294>
- (3) *Celiac Support Association Recognition Seal Program*. http://www.csaceliacs.info/csa_recognition_seal.jsp
- (4) *Gluten-Free Certification Organization (GFCO)*. <https://www.gluten.net/programs/industry-programs/gluten-free-certification-organization/>
- (5) Curtis, M. *Gluten Free Certification Program (GFCP)*. <http://www.gf-cert.org/>
- (6) *Codex Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten* (1979) Codex Alimentarius, Codex Standard 118–1979, rev. 2008, pp. 1–3. http://www.codexalimentarius.org/download/standards/291/cxs_118e.pdf
- (7) Leonard, M.M., & Vasagar, B. (2014) *Clin. Exp. Gastroenterol.* **7**, 25–37
- (8) Koerner, T.B., Abbott, M., Godefroy, S.B., Popping, B., Yeung, J.M., Diaz-Amigo, C., Roberts, J., Taylor, S.L., Baumert, J.L., Ulberth, F., Wehling, P., & Koehler, P. (2013) *J. AOAC Int.* **96**, 1033–1040. <http://dx.doi.org/10.5740/jaoacint.13-043>
- (9) Popping, B., Diaz-Amigo, C., & Hoenicke, K. (2010) *Molecular Biological and Immunological Techniques and Applications for Food Chemists*, Wiley, Hoboken, NJ, p. 368. <http://dx.doi.org/10.1002/9780470637685>