Validation Of Two Gluten Elisa Assays Utilizing A Novel Polyclonal Antibody System

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ABSTRACT

Celiac disease is an autoimmune disorder for which there is no cure, the only treatment being a lifelong abstention from a gluten containing diet. The FDA requires any food in the United States labelled gluten-free to contain less than 20 parts per million gluten as determined by validated analytical methods. The major sources of gluten in the American food stream are wheat, rye, and barley cereal grains, but the currently available methods do not detect gluten from these three grains equally. Bia Diagnostics, LLC has developed novel quantitative and qualitative immunoassays for the detection of gluten in foods, which are here validated following AOAC International guidelines. While the quantitative Enzyme Linked Immunosorbent Assay method failed to satisfy the AOAC Standard Method Performance Requirement 2017.01, the qualitative lateral flow device method meets the requirements of the AOAC Performance Tested Methods (PTM) program, granted certificate no. 011601 for the detection of gluten in buckwheat, chocolate syrup, soy milk, corn flakes, rinse waters, and on stainless steel surfaces. When used in conjunction with a management plan, it is suitable for use by food manufacturers to show compliance with gluten free labelling regulations.
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1.1 Gluten and Celiac Disease

While homo sapiens originally evolved as hunter-gatherers eating a diet of meats, fruits, and vegetables, humans domesticated gluten-containing cereal grains over 10,000 years ago in the Fertile Crescent (Avni et al., 2017) and civilizations have been relying on their nutritive properties ever since. These farmers realized that certain cereals (especially wheat and barley), when fermented and ‘leavened’, would hold their airy shape and texture. While they did not know that this was a result of carbon dioxide released from yeast fermentation being trapped in the stretchy gluten fibers of the grain endosperm (Shewry, Halford, Belton, & Tatham, 2002), they recognized that the resulting product was larger and was more enjoyable to eat. The development of agriculture to produce reliable supplies of wheat and barley for beer and bread was in large part responsible for the rise of modern civilization, driving the transition to sedentary agrarian societies (Lev-Yadun, Gopher, & Abbo, 2000). Today, gluten containing grains are used in a variety of ways to enhance flavor and texture, becoming a staple of the industrial food system (Delcour et al., 2012).

Gluten is a storage protein found in cereal grains such as wheat, rye, barley, and their cultivars, providing an energy source for seeds during germination (Biesiekierski, 2017). The wheat kernel contains 8-15% protein, of which 85-90% is gluten (Wieser, 2007). The gluten protein is comprised of alcohol-insoluble glutelin and alcohol-soluble prolamin protein polymers, the latter containing long amino acid sequences rich in glutamine and proline (Schalk, Lexhaller, Koehler, & Scherf, 2017). More specifically,
wheat gluten contains low-molecular-weight (LMW) glutenin subunits (GS) and high-
molecular-weight (HMW) GS glutelins, and α/β-, γ-, ω1,2-, and ω5-gliadin prolams; rye gluten contains HMW-secalin-GS glutelins, and ω-, γ-40k, and γ-75k secalin prolams; barley gluten contains D-hordein-GS glutelins, and β-, C-, and γ-hordein prolams (Röckendorf et al., 2017). The Codex Alimentarius, a collection of internationally recognized standards for food safety set forth by the Food and Agriculture Organization of the United Nations and World Health Organization, defines gluten more simply as “a protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5 mol/L NaCl” (C.A. Commission, 2008).

Unfortunately, around 1% of the world population has celiac disease (CD), an autoimmune disorder which causes damage to the small intestine, and for which there is no cure. The only treatment for this disorder is lifelong abstention from gluten-containing foods (Butterworth & Los, 2019). This disease was first described by Samuel Gee in 1887, who termed it ‘coeliac affliction,’ and called for rest, fasting, and a fruit diet as treatment (Dowd & Walker-Smith, 1974). In 1924, Sidney Haas described a treatment of ten patients with CD where eight patients eating a banana diet were cured, and the other two patients left untreated died (Haas, 1924). The responsible antigen was not recognized until the 1940s, when Dr. Willem Karel Dicke identified wheat as the culprit (van Berge-Henegouwen & Mulder, 1993). Due to the war in Europe limiting supplies of bananas and other fruits, which had been the accepted treatment up to that time, rationing meant much more bread was ingested by CD patients. Dicke noticed that when they were admitted to hospital and put on a strict wheat-free diet their symptoms subsided, but
when they were released and went back on rationed foods, their symptoms returned (van Berge-Henegouwen et al., 1993).

Symptoms of CD are chronic and varied, but are broadly characterized by inflammation and atrophy of the small intestine, resulting in classic irritable bowel symptoms, nutrient deficiencies, and failure to thrive; if left untreated, it can ultimately lead to death (Ludvigsson & Murray, 2019). The mechanisms of CD are not completely understood. It seems to be a result of both environment (gluten intake) and predisposing genetic factors, being more present in those carrying human leukocyte antigen (HLA) gene types HLA-DQ2 and/or HLA-DQ8, with the HLA genotype contributing to the genetic risk for CD at 30-50% (Gujral, Freeman, & Thomson, 2012). The frequency of HLA-DQ2 in Caucasian and Western European populations has been estimated at 20-30%, with similarly high frequencies in Northern and Western Africa and the Middle East; the frequency of HLA-DQ2 then declines to the east, with low frequency in Southeast Asia, and being virtually absent in Japan (Cummins & Roberts-Thomson, 2009). HLA-DQ8 has a similar distribution, but is most common in Amerindian populations of South and Central America, where approximately 90% may display the celiac phenotype (Layrisse et al., 2001). Rates of celiac disease across the world generally follow this genetic distribution, affecting around 1% of the populations of North America, South America, Europe, Australia, the Middle East, and Africa, with extremely low levels in Asia (Gujral et al., 2012).

All of the previously mentioned glutenin and prolamin subunits of gluten have been shown to be harmful to CD sufferers, impairing structure and function of the gut mucosa, when tested both in vivo and ex vivo (Stern et al., 2001). Additionally, they have
been shown to be immunogenic in proliferation assays using T cells from CD patients (Ciclitira, Evans, Fagg, Lennox, & Dowling, 1984). When gluten is ingested by a CD sufferer, these gluten sub-units are only partially digested in the upper intestinal tract, and peptide derivatives such as α-gliadin remain intact to pass the epithelial barrier of the gut and reach antigen-presenting cells in the lamina propria, a thin layer of connective tissue forming part of the mucous membrane in the gastrointestinal tract (Rostom, Murray, & Kagnoff, 2006). This sets off an autoimmune response characterized by inflammatory action and progressive architectural damage to the mucosa, leaving the intestine less able to absorb nutrients.

Celiac disease diagnosis is based on the predisposing genetic factors HLA-DQ2/8 as well as positive biopsies for serological antibodies when eating a gluten containing diet, although there is a spectrum of CD that may present in various forms. The classic form may be triggered and diagnosed at any age in life, and is often characterized by intestinal crypt hyperplasia (where the grooves between villi deepen) and blunting or atrophy of the villi, leading to a decreased villi/crypt ratio with poor nutrient absorption capacity (Dickson, Streutker, & Chetty, 2006). There is also an atypical form characterized by positive serology tests and genetic factors, but with limited to no abdominal abnormalities or intestinal symptoms, but with associated conditions such as osteoporosis, anemia and infertility. Finally, there is a latent form characterized by the presence of predisposing genetic factors and possible positive serology, but normal intestinal mucosa (Ferguson, Arranz, & O'Mahony, 1993).

A landmark study published in 2003 illustrated just how prevalent the disease was. In this study, serum anti-gliadin and anti-endomysial (EMA) antibodies were
measured, and in EMA positive subjects, an intestinal biopsy was performed and CD associated transglutaminase IgA antibodies and human leukocyte antigen (HLA) haplotypes were determined (Fasano et al., 2003). A total of 13,145 subjects were screened; 4,126 not-at-risk individuals, and 9,019 individuals either affected by CD or having first or second degree relatives affected by it. The results showed that CD occurs not only in patients displaying gastrointestinal symptoms, but often in first and second degree relatives of patients not displaying symptoms, indicating it is a much more common yet under-diagnosed disorder than previously thought (Fasano et al., 2003). Indeed, rates of CD appear to be rising all the world over as the western diet is adopted globally, suggesting that many people may be genetically predisposed to the disease but only present clinically when there is sufficient gluten in their diet (Cataldo & Montalto, 2007).

In addition to celiac disease, many more people (up to 10% of the population) have what is considered non-celiac gluten sensitivity. These patients do not have CD but for a variety of reasons feel better when gluten is removed from their diet (Gibson, Skodje, & Lundin, 2017). As there are no diagnostic biomarkers and much clinical uncertainty surrounding diagnosis, non-celiac gluten sensitivity is considered a set of symptoms caused by ingesting gluten which disappear on a gluten-free diet and occur in patients who have neither wheat allergies or CD (Fasano, Sapone, Zevullos, & Schuppan, 2015). Perhaps as a reaction to the increased visibility of gluten intolerance in the mainstream media, many individuals with neither CD nor non-celiac gluten sensitivity have chosen to adopt a gluten free diet to varying degrees. According to a 2015 GALLUP poll, 21% of Americans include gluten free foods in their diet (Riffkin, 2015), although a
separate poll in 2015 found only 8% of consumers buying gluten free products do so because of a gluten sensitivity (Hartman, 2015). Many people believe that eating gluten free foods can lead to weight loss, although there is little evidence to show that this is the case (Gaesser & Angadi, 2012); in fact, studies have shown that processed gluten free foods often contain a greater density of fat and sugar than their non-gluten free equivalents (Kulai & Rashid, 2014), and obesity and new onset insulin resistance have been identified in individuals after initiating a gluten free diet (Kabbani et al., 2012).

Regardless of the questionable health benefits, the large prevalence of consumers with either CD, non-celiac gluten sensitivity, or a perceived notion that a gluten free diet is somehow healthier has led to a boom in the gluten-free foods market, which has created more demand for standards and regulation.

1.2 Gluten Free Regulation
Avoiding gluten-containing foods has been the prescribed treatment for CD since the 1940s (Koehler, Wieser, & Konitzer, 2014), but options historically were limited to naturally gluten-free foods. The industrial food system did not cater largely to the gluten-free market until the early 2000s, when, as described above, it became obvious that more people either had latent CD or non-celiac gluten sensitivity (Zarkadas et al., 2006).

In response to this growth in demand, more processed gluten-free foods were put on the market, from breads and cereals to cookies and pretzels, many which used oat, corn, or rice flour and replaced the gluten with fats and gums to simulate the texture and flavor attributes (Gallagher, Gormley, & Arendt, 2004). Still, it was not uncommon for
products to be contaminated with wheat, rye or barley flour from the comingled agricultural and manufacturing processes (Thompson, Lee, & Grace, 2010).

In 2004, the United States Congress passed the Food Allergen Labelling and Consumer Protection Act, 21 USC 301 Note (FALCPA), in response to the finding that nearly 2% of adults and 5% of children in the US suffer from food allergies (US Food and Drug Administration, 2004). This bill requires food manufacturers to call out specific allergens in food labels when they are present, but does not set threshold levels for cross-contamination (Thompson, Kane, & Hager, 2006). Additionally, while it did recognize wheat as an allergen, it did not initially recognize rye or barley, or specifically call out gluten, as CD is a chronic autoimmune disorder and not an acute allergic reaction; it did however include a provision that the FDA issue a proposed rule to define and permit the voluntary use of the term ‘gluten-free’ on the labelling of foods (Thompson et al., 2006).

In 2013, the FDA declared that any food labelled ‘gluten-free’ must contain no more than 20 parts per million (ppm) gluten and must not contain any wheat, rye, barley, or their cultivars (such as triticale) as ingredients (US Food and Drug Administration, 2013). The 20 ppm threshold was chosen to reflect the lowest analytical level that could reliably be detected at the time using scientifically validated methods (US Food and Drug Administration, 2018), and which was considered protective for the majority of CD sufferers (Akobeng & Thomas, 2008). The ruling applies to all foods regulated by the FDA, such as packaged foods, dietary supplements, fruits, vegetables, and fish, and is voluntary; a gluten-free food does not have to be labelled as such, but a food labelled ‘gluten-free’ must meet these requirements (US Food and Drug Administration, 2013).
Manufacturers would have until August 2014 to bring their labels into compliance (US Food and Drug Administration, 2013).

The ruling does not apply to foods regulated by the US Department of Agriculture such as meat and poultry, or products regulated by the Alcohol and Tobacco Tax and Trade Bureau, such as distilled spirits and beers made with malted barley and hops (US Food and Drug Administration, 2018). However, as most gluten-free beers are made with a blend of gluten-free grains, such as corn, rice, or sorghum, these products are regulated by the FDA. Recognizing the analytical challenges posed by the fermentation process, which breaks down protein epitopes beyond recognition of the validated test kits, the FDA requires gluten free beer to contain less than 20 ppm gluten before fermentation, and that the manufacturer adequately evaluate the process for any potential gluten cross-contact (US Food and Drug Administration, 2017). While some research has shown that fermentation can reduce the toxicity of wheat, rye, and barley gluten on celiac patients (Kerpes, Fischer, & Becker, 2017), beer made with gluten-containing grains is still prohibited from being labelled ‘gluten-free’ (US Food and Drug Administration, 2017).

Many other countries have passed similar rulings on the use of gluten-free labelling on foods. In Canada, the Canadian Food Inspection Agency requires foods labelled ‘gluten-free’ to contain no gluten-containing grains (including their cross-bred varieties such as triticale, spelt, or kamut) as ingredients, and any gluten present from cross-contact must be below 20 ppm, as per B.24.018 of the Food and Drug Regulations (Canada, 2012). In Europe, the European Commission Implementing Regulation No 828/2014 allows for ‘gluten-free’ labelling on foods containing less than 20 ppm gluten, or ‘very low gluten’ labelling on foods containing one or more gluten containing
ingredient, but which have been processed to contain less than 100 ppm gluten (European Commission, 2014). In Australia and New Zealand, the Food Standards Code requires foods labelled ‘gluten-free’ to contain no detectable gluten, and foods labelled ‘low-gluten’ to contain less than 20 ppm (New Zealand, 2008). In Argentina, the Argentinian Food Code Article no. 1383 defines gluten-free foods as those containing less than 10 ppm gluten, and it requires the use of a national logo for labelling (Argentinian Food Code, 2011). These are the major countries, besides the US, with government policies for labelling foods ‘gluten-free’ (Celiac Disease Foundation, 2019).

Since the FDA made its decision on gluten-free labelling, the US gluten-free industry has shown little signs of slowing down, growing 136% from 2013 to 2015 (Reilly, 2016). With a wide range of options available, but many nutritional deficiencies to consider, many individuals adopting a gluten-free diet look to oats and oat-based products (Smulders et al., 2018). Oats are one of the healthiest whole grains, with five approved European Food Safety Authority health claims, relating to fiber content, favorable impact on cholesterol, blood glucose levels, and fecal bulk, as well as a high content of unsaturated fatty acids and polyphenols (Smulders et al., 2018).

There was initially some concern that avenins, the prolamin fractions of oats, could cause celiac symptoms, as there appear to be some CD sufferers with oat-avenin reactive T-cells in the mucosa of the small intestine (Arentz-Hansen et al., 2004). However, several follow-up studies have shown that oats do not contain any of the recognized gluten protein epitopes associated with celiac disease (Londono, 2013) and are safe for the vast majority of CD sufferers (Gatti et al., 2013; Kaukinen, Collin, Huhtala, & Mäki, 2013). While the Codex standard defines gluten as a protein from
wheat, rye, barley, or oats, it makes a special exception for oats, noting that ‘oats can be tolerated by most but not all people who are intolerant to gluten. Therefore, the allowance of oats that are not contaminated with wheat, rye or barley in foods covered by this standard may be determined at the national level’ (C.A. Commission, 2008).

Unfortunately, because the oat supply is largely comingled with other gluten-containing grains, it is extremely difficult to source truly gluten-free oats on an industrial scale (Hernando, Mujico, Mena, Lombardia, & Mendez, 2008). Additionally, oat “cleaning” procedures such as mechanical and hand-separating of grains are not 100% effective at removing gluten contamination from the oat supply (Koerner, Cléroux, Poirier, Cantin, Alimkulov, & Elamparo, 2011), and a study by the FDA in 2015 found that the presence of oats in otherwise gluten-free foods was strongly correlated with a gluten content of > 20 ppm (Sharma, Pereira, & Williams, 2015). Therefore, it is extremely important for food manufacturers to test for gluten contamination in oats at various stages of processing, or the final product may end up containing >20 ppm gluten, which can lead to costly losses and recalls. The challenge lies in implementing statistically significant sampling plans, as gluten can be extremely heterogeneously distributed in oats; to determine the overall gluten content of a batch, many analytical test portions must be taken, the actual number decreasing as you increase the sample size (Fritz, Chen, & Contreras, 2017). Due to the inherent challenge in sampling, the analytical methods employed become crucial to accurate measurement.

1.3 Gluten Detection Methods

Enzyme Linked Immunosorbent Assay (ELISA)-based methodologies are the industry standard for analytical gluten detection, due to their accuracy, relative ease of
use, and rapid run time (Haraszi, Chassaigne, Maquet, & Ulberth, 2011). First developed in 1971, ELISA is an analytical diagnostic technique utilizing either monoclonal or polyclonal antibodies to detect a target antigen (Engvall & Perlmann, 1972). The most common form of quantitative ELISA methodology is referred to as a ‘sandwich’, where purified antibodies specific to a target antigen are bound to test wells on a polystyrene microtiter plate (Sorell et al., 1998). An extracted liquid sample is then applied to the wells, and any antigenic protein that is present binds to the antibodies on the test plate. The sample solution is then washed off the plate, leaving only the target antigen bound to the antibodies, and a solution of antibodies conjugated to an enzyme (usually horseradish peroxidase, HRP) are then applied. If any antigen was present in the extracted sample solution, then the conjugated antibodies bind to the antigen, which is bound to the antibodies on the test plate, creating an antibody-antigen-antibody ‘sandwich’. The conjugate solution is then washed off, and a substrate is added (usually tetramethylbenzydine, TMB), which reacts with any HRP enzyme present to change color. This color change can then be measured as optical density by an absorbance plate reader and compared to a standard curve to quantify antigen in the sample.

Another antibody based immunochromatographic assay employed for qualitative gluten detection is the lateral flow device, or LFD (Scherf & Poms, 2016). These methods employ a dipstick, usually made of nitrocellulose paper, with a sample pad at the bottom containing gluten antibodies conjugated to colloidal gold particles (Koczula & Gallotta, 2016). When an extracted sample solution is applied to the sample pad, any gluten protein present binds to the conjugated antibodies and migrates across the test strip
(Koczula & Gallotta, 2016). At some point farther along the strip there is a test line made up of more antibodies specific to gluten, which bind to the gluten proteins already bound to the colloidal gold particles (Koczula & Gallotta, 2016). As more gluten is caught by the antibodies on the test line, more of the conjugated colloidal gold builds up and the test line becomes visible (Koczula & Gallotta, 2016). These tests are interpreted as a yes or no result, and have been shown to be effective at screening for gluten in a variety of food matrices, and by swabbing on surfaces such as stainless steel, at levels well below the FDA requirements for gluten free labelling (Lacorn et al. 2016). While LFDs are a rapid and useful screening tool for manufacturers, and can alert them to the presence of gluten contamination in their facility or product, quantitative ELISA methods are still the preferred method of verifying compliance to gluten-free labelling (Scherf & Poms, 2016).

Gluten ELISA methods have been commercially available from a variety of test kit manufacturers, such as R-biopharm, Romer Labs, ELISA Systems, and Neogen, since 1991 (Skerritt & Hill, 1991). The majority of these available test kits utilize monoclonal antibodies, which target different peptide sequences of the gluten protein and react with different intensities to gluten from wheat, rye, and barley grains, depending on what antigen they were developed against (Tranquet, Lupi, Echasserieau-Laporte, Pietri, Larré, & Denery-Papini, 2015). The Skerritt antibody was developed to detect the ω-gliadin fraction of wheat gluten, utilizing a simple ethanol extraction, and was adopted as the first Association of Official Analytical Chemists (AOAC) Official Method of Analysis for measuring gluten in foods (Skerritt et al., 1991). However, its low reactivity to gluten from barley and a lower detection limit of 150 ppm (Thompson & Mendez, 2008) has
caused it to fall out of favor for use in regulatory testing, though it is still commercially available from ELISA Systems.

The G12 antibody was developed to detect the 33-mer amino acid sequence of α-gliadin, a peptide that has been identified as one of the major contributors to gluten immunotoxicity (Moron et al., 2008). Romer Labs Agraquant Gluten G12 test kit is also an AOAC Official Method of Analysis, utilizing a reducing mercapto-ethanol-based extraction validated for rice-based food products, with a limit of detection of 5 ppm (Halbmayr-Jech, Rogers, Don, & Prinster, 2015). However, it also reacts to some species of oats (Slot, van der Fels-Klerx, Bremer, & Hamer, 2016), making it less desirable when testing presumably gluten-free oat products for the presence of gluten from other sources, as it can potentially give false positive results.

The R5 antibody was developed to detect the key toxic amino acid pentapeptide sequence QQPFP, present in gliadins, secalins, and hordeins from wheat, rye, and barley (Kahlenberg et al., 2006), and the reducing mercapto-ethanol based ‘Mendez’ extraction was developed in conjunction to extract gluten from processed foods (Osman et al., 2001); as such it has gained wide acceptance in the international regulatory community (Thompson et al., 2008). Since 2008, the Codex Alimentarius Commission of the United Nations/World Health Organization has deemed test kits using the R5 antibody and ‘Mendez’ extraction system to be Type 1 methods for gluten detection. Type 1 methods provide the most internationally acceptable measures of analytes in foods, being defined as ‘a method which…serves by definition as the only method for establishing the accepted value of the item measured’ (C.A Commission, 2008).
While there are two commercially available R5 based test kits for gluten, (Neogen Veratox Gluten ELISA and R-biopharm R7001 RIDASCREEN Gliadin ELISA), both with a lower limit of detection of 5 ppm, the R7001 test kit is the only method meeting this criteria that is an AOAC Official Method of Analysis, for quantifying gluten in corn and rice based products (Immer & Haas-Lauterbach, 2012). It is also an American Association of Cereal Chemists (AACC)-approved method for measuring intact gluten (Koehler et al., 2013), and has become the Codex endorsed method (C. A. Commission, 2013).

While the R7001 kit does an excellent job of accurately quantifying gluten from wheat, to which it is calibrated, it overestimates gluten from rye and barley grains by 300-400%, as the QQPFP pentapeptide sequence it recognizes is present in higher abundance in their gluten proteins (Lexhaller, Tompos, & Scherf, 2017). In oats, this can result in an artificially high, false positive result of >20 ppm gluten contamination if the gluten is derived from rye or barley (Kanerva, Sontag-Strohm, Ryöppy, Alho-Lehto, & Salovaara, 2006). Additionally, gluten contamination in oats is very heterogeneous, and the R7001 test kit only calls for a one gram analytical test portion, which is not large enough to determine the true gluten content of a batch of any considerable size (Fritz, Chen, & Contreras, 2017). To account for this, food manufacturers testing oats for gluten have to perform several sample replicates to obtain a mean gluten value of the batch (with the number of replicates going up correspondingly with batch size).

In response to these shortcomings, the AOAC launched a working group to develop a Standard Method Performance Requirement (SMPR) for the quantification of gluten in oats, the result of which was SMPR 2017.021: Quantitation of Wheat, Rye, and
Barley Gluten in Oats (Boison et al., 2018), opening the way for gluten test kits utilizing other antibody/extraction systems to gain acceptance in the scientific and regulatory communities.
2.1 Assay Development Overview

To fill the gaps in gluten detection previously described and to meet the demands of regulators and a complex, varied food industry, Bia Diagnostics has developed a commercial quantitative gluten ELISA, referred hereto as the Bia Diagnostics Gluten Assay. This test has been validated against AOAC SMPR 2017.021 requirements (Table 1), as well as the general AOAC validation procedures for quantitative gluten ELISA methods. (Abbott et al., 2010) It utilizes a novel polyclonal antibody and a proprietary extraction system, which together give a more balanced response to gluten from wheat, rye, and barley. Additionally, it calls for a five gram sample size and is calibrated to the most well characterized gluten material available, Prolamin Working Group (PWG) gliadin, a purified extraction of wheat gliadin which has become the standard reference material for gluten quantification (Eckert et al., 2006), using the standard 2x factor to convert gliadin concentration to gluten concentration (Herbert Wieser & Koehler, 2009). Total test time from extraction to results is 90 minutes, and the kit has a quantitative range of 5-80 ppm gluten, detecting well below the FDA threshold of 20 ppm and satisfying the SMPR requirements for analytical range (Table 1). This chapter further describe the parameters and performance of this assay.

Table 1. AOAC SMPR 2017.021 Requirements

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical Range, ppm</td>
<td>≤5 to ≥15</td>
</tr>
<tr>
<td>LOQ, ppm</td>
<td>≤5</td>
</tr>
<tr>
<td>LOD, ppm</td>
<td>≤5</td>
</tr>
<tr>
<td>Recovery, %</td>
<td>50 to 200</td>
</tr>
</tbody>
</table>
2.2 Materials and Methods

Materials:
Polyclonal chicken antibodies raised against the α-gliadin prolamin fraction of wheat gluten and coated to 96-well microtiter plates were provided by 3M Food Safety (St Paul, MN). The same antibodies were conjugated to horseradish peroxidase (HRP) enzyme and concentrated to a 10x solution. Tests plates, conjugate solution, sample diluent buffer, wash buffer, and stop solution were all provided by 3M Food Safety (St Paul, MN). Spiked oat samples were obtained from Medallion Labs (Minneapolis, MN), as part of the AOAC Gluten in Oats SMPR program. Purified gliadin for the test calibration was obtained from the Prolamin Working Group (PWG) (Leiden, The Netherlands). Commercially available R7001 RIDASCREEN Gliadin test kits were obtained from R-biopharm (Darmstadt, Germany). All other materials, including naturally contaminated food samples and 10x concentrated extraction solution, were provided by Bia Diagnostics (Colchester, VT).

Methods:
Spiked oat samples were analyzed by the Bia Diagnostics Gluten Assay and results were compared to the sample’s assigned values (determined at Medallion Labs by wet chemical protein method) (Wehling & Scherf, 2019). All other food samples were analyzed by the Bia Diagnostics Gluten Assay and the R-biopharm R7001 method. While there are no reference values to compare these results to, the R7001 method is an AOAC Official Method of Analysis and CODEX Type I method for measuring gluten in processed foods, so it was used as a reference. The R7001 test method was run following
its Instructions for Use. Both methods were run at Bia Diagnostics laboratories in
Colchester, VT, under ISO 17025 accredited conditions.

**Bia Diagnostics Gluten Assay Sample Preparation:**
For each sample being analyzed, 100 grams was homogenized in a Cuisinart Mini-Prep
food processor model no. DLC-2A until well mixed. From these homogenized samples,
five gram test portions were weighed out on an analytical balance (Mettler Toledo,
Columbus, OH) into 50 ml conical tubes (Celltreat, Pepperell, MA). To each test portion,
45 mls of room temperature (20-25°C) 1x proprietary extraction solution was added, and
the sample was vortexed (VWR, Radnor, PA) until well mixed before being put in a
shaking incubator (Labline, Asheville, NC) at 60°C for 30 minutes at 150 rpm. The
samples were then centrifuged for 2 minutes at 3,500 \( x \) g (Eppendorf, Hauppauge, NY)
and allowed to cool to room temperature. To create the calibrator solution, PWG gliadin
was dissolved in 60% ethanol (Labchem, Zelionople, PA) to a final concentration of 40
\( \mu \)g/ml gliadin, corresponding to 80 \( \mu \)g/ml gluten. To match the sample extraction process,
0.5 ml of this calibrator solution was extracted with 4.5 ml of proprietary extraction
solution and incubated at 60°C for 30 minutes. After cooling to room temperature, this
solution was diluted an additional 1/50 (20\( \mu \)l/980\( \mu \)l) in sample diluent buffer, for a final
1/500 dilution ratio at a concentration of 160 ng/ml gluten (corresponding to 80 ppm
gluten in original sample). Linear 1:1 dilutions of this 160 ng/ml solution were made in
diluent buffer, to create a standard curve down to 10 ng/ml (correlating to 5 ppm gluten
in original sample).
Bia Diagnostics Gluten Assay Test Implementation:
Duplicate 1:50 dilutions were made from each sample extract into sample diluent buffer. For this, 20 µl was taken from the aqueous layer and added to 980 µl of diluent buffer in a clean 2 ml centrifuge tube (USA Scientific, Orlando, FL). Samples were now at a 1/500 total dilution, matching the dilution ratio of the calibrators. To each test well, 100 µl of calibrator or sample was added, and the plate was incubated for 30 minutes at room temperature. Following this incubation, the plate was washed on a Biotek ELX50 automated plate washer (Biotek, Winooski, VT), after which 100 µl of enzyme-conjugated antibody solution was added to each test well. The test plate was incubated for 10 minutes at room temperature (20-25°C), followed by another wash cycle. After washing, 100 µl of chromogenic substrate solution (SeraCare TMB SureBlue Reserve, Milford, MA) was added to each test well. The plate was incubated for a final 10 minutes, at which time 100 µl of 0.3M nitric acid stop solution was added to each test well to stop the reaction. The plate was read on a Biotek ELx800 absorbance plate reader at 450nm, and data reduction was done by Biotek Gen5 software, using a 4-parameter fit non-linear regression algorithm to calculate the sample results from the generated standard curve. Duplicate sample results were averaged.

2.3 Results
Cross-Reactivity Study
A panel of 37 gluten-free labelled matrixes were quantitatively pre-screened for natural gluten contamination by the reference method, and each was assayed by the Bia Diagnostics Gluten Assay to test for cross-reactivity. As shown in Table 2, the results show no cross-reactivity of this antibody system to any of the screened compounds.
Table 2. Cross-reactivity results

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Cross-Reactivity Results (ppm)</th>
<th>Matrix</th>
<th>Cross-Reactivity Results (ppm)</th>
<th>Matrix</th>
<th>Cross-Reactivity Results (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coconut Flour</td>
<td>&lt;5</td>
<td>Soya Flour</td>
<td>&lt;5</td>
<td>Lima Bean Flour</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Yellow pea Flour</td>
<td>&lt;5</td>
<td>Coffee</td>
<td>&lt;5</td>
<td>White Bean Flour</td>
<td>&lt;5</td>
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<td>Meat</td>
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<td>Xanthan Gum</td>
<td>&lt;5</td>
<td>Flax seed flour/meal</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Sorghum Flour</td>
<td>&lt;5</td>
<td>Arrowroot</td>
<td>&lt;5</td>
<td>Chestnut Flour</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Garfava Flour</td>
<td>&lt;5</td>
<td>Hazelnut Flour</td>
<td>&lt;5</td>
<td>Guar Gum</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Tapioca Flour/Starch</td>
<td>&lt;5</td>
<td>White Rice Flour</td>
<td>&lt;5</td>
<td>Almond Gum</td>
<td>&lt;5</td>
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<tr>
<td>Green Pea Flour</td>
<td>&lt;5</td>
<td>Quinoa Flour</td>
<td>&lt;5</td>
<td>Corn Starch/meal</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Black bean Flour</td>
<td>&lt;5</td>
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<td>&lt;5</td>
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<td>Dried Fruits</td>
<td>&lt;5</td>
<td>Fava Bean Flour</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Potato flour/starch</td>
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<td>Buckwheat Flour</td>
<td>&lt;5</td>
<td>Guar Gum</td>
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<td>Lentil Flour</td>
<td>&lt;5</td>
<td>Millet Flour</td>
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<tr>
<td>Egg Powder</td>
<td>&lt;5</td>
<td>Brown Rice Flour</td>
<td>&lt;5</td>
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<td></td>
</tr>
</tbody>
</table>

Accuracy Study
Spiked oat flour samples obtained from General Mills as part of the Gluten in Oats SMPR program were tested on twelve separate days over the course of six months to determine the mean recovery and variability in results. As seen in Table 3 and Figure 1, the mean recoveries were acceptable to the SMPR parameters of 50-200% accuracy for oat flour spiked with 10 ppm and 20 ppm wheat gluten, as well as oat flour spiked with 10 ppm and 20 ppm rye gluten and 10 ppm barley gluten; however, recovery was too low for oat flour spiked with 20 ppm barley gluten. When taking standard deviation into account, recovery was too high for oat flour spiked with 10 ppm rye gluten.
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<th>8.4</th>
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<th>7.3</th>
<th>7.6</th>
<th>8.7</th>
<th>10.2</th>
<th>7.8</th>
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</tr>
<tr>
<td></td>
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<td>0.3</td>
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<td>0.7</td>
<td>0.2</td>
<td>0.2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table 3: SMP Sample Results
Incurred Food Matrix Study
A panel of processed foods contaminated in processing were screened by the R7001 reference method for gluten contamination. Additionally, gluten-free muffin, brownie, and chocolate chip cookie batters were spiked with 10 ppm PWG gliadin (20 ppm gluten) before baking, thoroughly processed, and screened by the R7001 reference method (see Appendices 1-3 for recipes). All samples were then tested by the Bia Diagnostics Gluten Assay. Results, as seen in Table 4 and Figure 2, show recoveries generally below 50%, with the exception of the less processed samples, such as coffee cake mix, rice flour, and celery spice. Recovery was also >50% for a few of the processed matrixes such as the cranberry almond and double chocolate cookies as well as the lentil chips; however, it was <33% for the 20 ppm spiked, baked matrixes.
### Table 3. Incurred Sample Results

<table>
<thead>
<tr>
<th>Sample</th>
<th>R7001 reference Method Value (ppm)</th>
<th>Result (ppm)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranberry Almond Cookie</td>
<td>15.5</td>
<td>9.3</td>
<td>60.0</td>
</tr>
<tr>
<td>Double Chocolate Cookie</td>
<td>11.2</td>
<td>8.8</td>
<td>78.6</td>
</tr>
<tr>
<td>Chocolate Banana Chips</td>
<td>7.1</td>
<td>2.8</td>
<td>39.4</td>
</tr>
<tr>
<td>Cinnamon Coffee Cake Mix</td>
<td>14.6</td>
<td>10.1</td>
<td>69.2</td>
</tr>
<tr>
<td>Macaroon</td>
<td>20</td>
<td>9.2</td>
<td>46.0</td>
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<tr>
<td>Chocolate Coconut Cookie</td>
<td>10</td>
<td>4.3</td>
<td>43.0</td>
</tr>
<tr>
<td>20ppm PWG cookie</td>
<td>10.9</td>
<td>3.4</td>
<td>31.2</td>
</tr>
<tr>
<td>20ppm PWG brownie</td>
<td>9.1</td>
<td>2.6</td>
<td>28.6</td>
</tr>
<tr>
<td>20ppm PWG Muffin</td>
<td>12.9</td>
<td>2.9</td>
<td>22.5</td>
</tr>
<tr>
<td>Lentil Chips</td>
<td>11.5</td>
<td>6.7</td>
<td>58.3</td>
</tr>
<tr>
<td>Choc chip cookie</td>
<td>4.9</td>
<td>1.4</td>
<td>28.6</td>
</tr>
<tr>
<td>Rice Flour</td>
<td>18.9</td>
<td>11.6</td>
<td>61.4</td>
</tr>
<tr>
<td>Celery Spice</td>
<td>3.3</td>
<td>6.5</td>
<td>197.0</td>
</tr>
</tbody>
</table>
The lower limit of detection of an assay is the lowest concentration of analyte that can be distinguished from a true blank, or zero analyte concentration sample. To estimate this, thirty blank samples (extraction buffer diluted 1/50 in diluent buffer) were run on the same test plate, with a quantitative standard curve run in duplicate. The mean and standard deviation of the results were calculated, and three standard deviations above the mean was determined to be the lower limit of detection of the assay. Based on this model, the lower limit of detection of the Bia Diagnostics Gluten assay is 0.05 ppm.

The quantitative range of the assay was set between 5 ppm and 80 ppm to satisfy the requirements of SMPR 2017.021, and to be well below the 20 ppm regulatory threshold set by the FDA.

Figure 2. Incurred Sample Results
2.4 Discussion
The polyclonal antibody and proprietary extraction system used together in the Bia Diagnostics Gluten assay accurately quantitate wheat gluten in oats, as the antibody was developed against α-gliadin, and the kit is calibrated to PWG gliadin, both derived from wheat. However, they underestimate barley gluten and overestimate rye gluten, putting the assay outside the acceptable range of 50-200% accuracy outlined in SMPR 2017.021. When testing naturally contaminated processed samples and incurred samples created in the lab, in many cases the assay had <50% recovery compared to the R7001 reference method; more study is needed to determine its reactivity to various levels of processing on gluten from wheat, rye, and barley, and to specific glutelin and prolamin subunits. It should be noted that the incurred samples had low recovery vs theoretical concentrations on the R7001 reference method as well, likely due to the PWG gliadin being broken down by the effects of heat processing beyond recognition of the R5 antibody, and that the R7001 test method also fails to meet the requirements of SMPR 2017.021. (Wehling et al., 2019) The Bia Diagnostics Gluten assay, with its large sample size and accuracy to wheat gluten, is best suited for testing samples which have not been heavily processed and are likely to be heterogeneously contaminated with wheat.

Food manufacturers using quantitative ELISA methods to verify compliance with FALCPA gluten-free labelling regulations should currently test their products with AOAC Official Methods of Analysis 2012.01 or 2014.03, which utilize the R5 and G12 antibodies, respectively. While both methods are flawed and fail to meet the requirements of SMPR 2017.021, they have both gained regulatory acceptance, and when used in conjunction with a gluten-free management program can provide confidence in the safety of ‘gluten-free’ labelled products for gluten-sensitive consumers.
2.5 Conclusions
The Bia Diagnostics Gluten assay is a fast method for quantifying gluten from wheat, rye, and barley in heterogeneous samples. While it is calibrated and accurate to wheat gluten in oats, it underestimates barley gluten in oats by more than 50% and overestimates rye gluten in oats by more than 200%. This method fails to meet the requirements of AOAC SMPR 2017.01 Quantitation of Wheat, Rye, and Barley Gluten in Oats.
CHAPTER 3. BIA DIAGNOSTICS QUALITATIVE GLUTEN ASSAY

3.1 Method Overview
To meet the demands of food manufacturers looking for rapid pass/fail screening results, Bia Diagnostics has developed a qualitative gluten Lateral Flow Device (LFD). This test has been validated against AOAC Research Institute Performance Tested Methods (PTM) requirements, as well as the AOAC Validation Guidelines for Qualitative Binary Chemistry Methods (AOAC, 2013). It utilizes a novel polyclonal antibody and proprietary extraction system, which together detect gluten reliably down to 5 ppm in bread, buckwheat, chocolate syrup, dry cereal, and pasteurized soy milk matrixes as well as clean in place (CIP) or rinse water solutions, and on environmental surfaces down to 5 µg/ml/100cm². The method has a 3 minute extraction time and 11 minute incubation time to results, and is interpreted visually.

3.2 Materials and Methods
Materials:
Lateral flow device test strips utilizing polyclonal antibodies raised against the α-gliadin prolamin fraction of gluten were provided by 3M Food Safety (St Paul, MN). Purified gliadin for spiking samples was obtained from the Prolamin Working Group (PWG) (Leiden, The Netherlands). The R-biopharm R7001 quantitative ELISA method was used as a reference measurement. All other commodities were obtained at Shaw’s supermarket (Colchester, VT).
Methods:
The test procedure consists of a three minute room temperature extraction of 0.2 grams of a sample, or swabbing of a 100 cm² surface which does not require an incubation period. Extraction solution (100 µl) is then added to the application area of the test strip, which spreads by capillary action across the LFD strip. The test is interpreted visually after 11 minutes, with the presence of two red lines (test and control) indicating a positive result. One red line (control) indicates a negative result. If no control line develops the test should be considered invalid. Validation parameters included testing cross-reactivity, interference, product consistency, stability, robustness, incurred samples and food matrixes. Multiple reference materials were used as spiking sources in this validation to ensure the method is sensitive to more than one type of gluten contamination.

Sample Preparation:
For liquid raw ingredients and finished products, 900 µl of extraction/running buffer was measured into a 2.2 ml dilution tube. From a well-mixed representative sample, 100 µl was added to the tube and mixed well. One LFD was removed from its pouch and placed on a clean flat surface, and 100 µl of extracted sample was added to the sample application area. Results were read visually at 11 minutes.

For non-liquid raw ingredients and finished products, a representative sample was ground into a homogenous fine mixture. From this, 0.2 g was measured into a 2.2 ml dilution tube, and 1.8 ml of extraction/running buffer was added before mixing well for a minimum of 3 minutes. The sample was then centrifuged 20-30 seconds at 5000-7000 RPM. One LFD was removed from its pouch and placed on a clean flat surface, and 100 µl of extracted sample was added to the sample application area. Results were read visually at 11 minutes.
For chocolate samples, 500 µl or 0.5 g of well-mixed sample was measured into a 2.2 ml dilution tube, and 500 µl of warm (~60°C) extraction/running buffer was added to the tube and mixed well for ~30 seconds. Into a second clean 2.2 ml dilution tube, 900 µl of extraction/running buffer was added. From the first tube, 100 µl was taken and added to the second tube, mixed well for ~30 seconds, and then centrifuged for 20-30 seconds at 5,000-7,000 rpm. One LFD was removed from its pouch and placed on a clean flat surface, and 100 µl of extracted sample from the second tube was added to the sample application area. Results were read visually at 11 minutes.

For swab samples, 500 µl of extraction/running buffer was measured into a 2.2 ml dilution tube. The tip of a clean cotton swab was dipped into the tube, wetting the tip with buffer. The 100 cm² surface to be tested was surveyed by moving the swab back and forth on the area of interest. The swab was inserted back into dilution tube, swirled several times to remove protein into solution, and the tip was broken off into the tube with the cap closed. One LFD was removed from its pouch and placed on a clean flat surface, and 100 µl of extracted sample was added to the sample application area. Results were read visually at 11 minutes.

For Clean in Place (CIP) solution, 800 µl of extraction/running buffer was measured into a 2.2 ml dilution tube. Into this tube, 200 µl of CIP solution was added and mixed well for ~30 seconds. One LFD was removed from its pouch and placed on a clean flat surface, and 100 µl of extracted sample was added to the sample application area. Results were read visually at 11 minutes.

Preparation of the Validation materials:
Food matrixes used included Silk Soy Milk, Nature’s Path Honey Corn Flakes, Bob’s Red Mill Buckwheat Flour, and Ah!Laska Organic Chocolate Syrup, all of which were
labelled Gluten Free and screened by reference method R7001 for natural contamination. Clean in Place solution, pH 8.20 was obtained from a major soft drink manufacturer for use in the environmental surface testing study. Reference Materials used were PWG gliadin, gliadin content 88.2% (by Certificate of Analysis), and King Arthur Flour 100% whole wheat flour (WWF), gluten content 15% as measured by R7001.

For the food matrix study, a 400 ppm gluten working solution was created by dissolving 0.1 grams of PWG gliadin in 400 ml 60% ethanol. A 200 ppm working solution was created by diluting 100 ml of the 400 ppm solution in 100 ml 60% ethanol. A 100 ppm working solution was created by diluting 100 ml of the 200 ppm solution in 100 ml 60% ethanol. A 70 ppm working solution was created by diluting 70 ml of the 100 ppm solution in 30 ml 60% ethanol. A 20 ppm working solution was created by diluting 10 ml of the 100 ppm solution in 40 ml 60% ethanol.

Spiking of the test portions was achieved by weighing 0.2 gram aliquots of well-homogenized sample into 2.2 ml dilution tubes and spiking each tube with 10 µl of the appropriate PWG working solution (i.e. 10 µl of the 400 ppm solution for a 20 ppm spiked sample). Spiking of the 0 ppm test portions was achieved by weighing out 0.2 gram aliquots of well-homogenized sample into 2.2 ml dilution tubes and spiking each tube with 10 µl of 60% ethanol. Each tube was vortexed, blind-coded and randomized, and let to sit for an hour before being analyzed by LFD. For each contamination level, 0.25 gram sample portions were added to three 50 ml tubes, each was spiked with 12.5 µl of appropriate spike solution, blind-coded, randomized and analyzed by R7001 to ensure accurate spiking levels.

For the stability, lot-to-lot variability, test kit variation, and ruggedness studies, a
750 ppm working solution of WWF was created by extracting 0.5 grams in 100 ml 60% ethanol. Spiking of white rice flour at 15 ppm was achieved by weighing out 0.2 gram aliquots into 2.2 ml tubes and adding 4 µl of the 750 ppm working solution to each tube. Blank samples were spiked with 4 µl of 60% ethanol. Samples were then blind-coded, randomized and allowed to sit for an hour before being analyzed by LFD.

For the stainless steel surface swabbing study, a 20 ppm PWG working solution was created by diluting 10 ml of the 100 ppm PWG working solution from the food matrix study with 40 ml of 60% ethanol. A 2.5 ppm PWG working solution was created by diluting 6.25 ml of the 20 ppm working solution with 43.75 ml of 60% ethanol. For each replicate of each inoculation level, 1 ml of working solution was added to a 10 cm x 10 cm stainless steel square, allowed to dry overnight, and tested by LFD according to the swabbing procedure.

3.3 Results
Cross-reactivity and Interference Study
A panel of 37 gluten-free labelled matrixes were quantitatively pre-screened for natural gluten contamination by the reference method, and each was assayed by LFD to test for cross-reactivity. As shown in Table 5, the results show no cross-reactivity with any of the screened compounds, with the exception of Oat Flour which showed a faint test line; upon re-running 30 replicates, all were negative, indicating that the slight test line was a false positive result. Each of the pre-screened matrixes used in the cross-reactivity study were then spiked with PWG gluten at 10 ppm and assayed by LFD. As shown in Table 4, 28/30 initially showed positive results. Milk Powder, which initially had a negative response, was re-run with 30 replicates and showed all positive results, indicating that the
first result was a false negative. Xanthan Gum, a very hygroscopic ingredient, had to be
diluted an extra 1/10 to be analyzed successfully and thus contained only 1 ppm gluten,
which could not be successfully analyzed at this low level.

Table 4. Cross-reactivity and interference results

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Cross-Reactivity Results (+/-)</th>
<th>Interference Results (+/-)</th>
<th>Matrix</th>
<th>Cross-Reactivity Results (+/-)</th>
<th>Interference Results (+/-)</th>
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<tr>
<td>Coconut Flour</td>
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<td>Soya Flour</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Yellow pea Flour</td>
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<td>Coffee</td>
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<td>Meat</td>
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<td>+</td>
<td>Xanthan Gum</td>
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<td>Sesame Flour</td>
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<td>Flax seed flour/meal</td>
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<td>-</td>
<td>+</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*Initial result negative, subsequent 30 re-test results positive

^Initial result positive, subsequent 30 re-test results negative
Consistency and Stability Studies

Three different production lots of LFDs were tested for lot-to-lot variability (lots C11N-01A, C25N-01A and I16N-01A). In this study, 30 replicates of rice flour were spiked with blank solution (60% ethanol), and 30 replicates were spiked with WWF solution at 15 ppm gluten. Three sets of 20 replicates (ten blank and ten 15 ppm spiked replicates each) were blind-coded and randomized, and each set of 20 replicates was tested by a different lot of LFDs. Using this as baseline data for the accelerated stability study, one lot of LFDs was stored at 25°C and tested at 10, 20, 32 and 50 days with ten negative and ten spiked positive rice flour samples. As shown in Table 6, all non-spiked samples had negative results while all samples spiked at 15 ppm had positive results, with all lots and at all time points.

Table 5. Variability and stability results

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Spike concentration ppm</th>
<th>Number of replicates</th>
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<th>Lot #</th>
<th>Days at 25°C</th>
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</thead>
<tbody>
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Robustness Study
Robustness was tested by varying several test parameters, including centrifuging vs not centrifuging the samples before application, applying 80 µl vs 120 µl to the test well, and reading the results at 3 minutes vs. 15 minutes. By combining these variables, 8 different conditions were tested in addition to the baseline method (centrifuging the sample, applying 100 µl to the sample application area and reading at 11 minutes). White rice flour was used as a sample matrix, and each variable was tested with ten negative (spiked with 60% EtOH) and ten positive (spiked with 10 ppm WWF) samples which had been extracted, blind coded and randomized. As shown in Table 7, the only unusual results occurred when reading the strips at 3 minutes after centrifuging and applying 120 ul to the sample application area, with two false negative results out of ten replicates.
Table 6. Robustness results

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<th>Matrix</th>
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Food Matrix Study
Soy milk, corn flakes, buckwheat flour and chocolate syrup were confirmed to be free of natural contamination by reference method R7001. Each of these matrixes was then spiked with PWG gluten at 0, 5, 10 and 20 ppm. Test samples were blind-coded, with 30 replicates being screened by LFD and three replicates analyzed by reference method R7001. Results for both the LFDs and reference method are presented in Table 8.
Probabilities of detection (PODs) were calculated for each spike level, defined as ‘the proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration’ (Wehling, LaBudde, Brunelle, & Nelson, 2011). All unspiked samples were negative, with PODs of 0.00. All samples spiked at 5 ppm, 10 ppm and 20 ppm were positive at 11 minutes, with PODs of 1.00.

Rice flour spiked at 10,000 ppm with WWF was also tested to determine if there was an overload, or “hook” effect at high levels of contamination. As shown in Table 8, this did not cause any false negative results.
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Incurred Sample Study
A gluten-free bread, and the same gluten-free bread spiked with 20 ppm PWG gluten were baked (see Appendix 4 for recipe). Both samples were frozen and ground into a fine particle size before being blind-coded and analyzed by both the reference method and LFD. As seen in Table 8, PODs for the negative bread samples were 0.00, and PODs for the positive samples were 1.00.

Fractional Recovery Study
To determine fractional recovery of the method, i.e. the level where the method gives 25-75% positive results, corn flakes and chocolate syrup were spiked at low levels with PWG spike solution and assayed by LFD. To eliminate subjectivity of test line results when read by eye (i.e. very low level positives vs negatives at the threshold level), test results were determined by a Qiagen ESEquant LFR reader (Qiagen, Hilden, Germany), with readings less than 25 milliVolts (mV) being negative and greater than 25 mV being positive; this mV level correlates to what we consider the threshold for a line visible to the naked eye. Results, as seen in Table 8, show that Corn Flakes spiked at 1 ppm had a POD of 0.63, while chocolate syrup spiked at 3.5 ppm had a POD of 0.27, indicating the method is more sensitive to gluten in corn flakes than in chocolate syrup. This is visually represented in Figure 3.

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Environmental Surface Testing Study
Clean in Place (CIP) rinse water was tested at 0 ppm, 5 ppm, and 10 ppm spiked concentrations with n=30 replicates per concentration by LFD, as well as n=3 replicates per concentration by R7001. Results, as seen in Table 8, show POD values of 0.00 for each non-spiked replicate, and PODs of 1.00 for each 5 and 10 ppm replicate. Additionally, stainless steel was tested by swabbing 100 cm² surfaces that had been coated with 1 ml each of either blank 60% ethanol (n=5), 2.5 ug/ml PWG gluten spike (n=30), or 5 ug/ml PWG gluten spike (n=5), and allowed to dry overnight. Test strip results for the 2.5 ug/ml surface tests were determined using the Qiagen ESEquant LFR reader to determine fractional recovery levels. Results, as seen in Table 8, show POD values of 0.00 for blank samples, 0.50 for 2.5 ug/ml/100cm², and 1.00 for 5 ug/ml/100cm² spiked samples.
3.4 Discussion

As a qualitative screening method, the Bia Diagnostics Gluten LFD met the requirements of the AOAC PTM program. There was no observed cross-reactivity with any of the tested matrixes, and the probabilities of detection for samples containing ≥5 ppm gluten in all of the tested matrixes was 1.00, with 30 replicates tested at each analyte/matrix concentration. On stainless steel surfaces coated with 5 ug/ml/100cm² the POD was 1.00, with 5 replicates tested. As this is a qualitative method, each matrix will have a different lower limit of detection (LLOD) somewhere between 0 and 5 ppm, where the probability of detection falls to between 0.25 and 0.75. In this validation, the LLOD was only determined for corn flakes and chocolate syrup, which had lower LLODs of 1 ppm and 3.5 ppm, respectively, and on stainless steel surfaces, with an LLOD of 2.5 ug/ml/100cm².

The method was stable across three production lots of test kits, and showed little variation in results when changing test parameters, such as centrifuging, sample volume applied to the strip, and incubation time. The only condition affecting the method was when the sample was centrifuged, with 120ul added to the test strip and results read after 3 minutes of incubation. This resulted in a lower POD of 0.8, indicating that the test should be incubated for a minimum of 10 minutes to reduce the risk of false negative results. High concentrations of gluten did not cause a hook, or overload effect, indicating the test has a large analytical range between 5 and 10,000 ppm.

The Bia Diagnostics Gluten LFD method is comparable to the AOAC OMA 2015.16, commercially available from R-biopharm as the R7003 RIDA Quick Gluten test, a qualitative method validated for testing processed and unprocessed corn-based products, as well as stainless steel surfaces (Lacorn et al. 2016). The RIDA Quick Gluten
method consistency detects gluten in unprocessed corn products down to 4.4 ppm with a 30 second ethanol-based extraction and 10 minute incubation period; down to 6.4 ppm in processed samples with a 100 minute mercapto-ethanol based extraction and 10 minute incubation period; and on stainless steel surfaces down to 2 ug/ml/100cm² with no extraction and a 5 minute incubation period. While this method is faster and more sensitive on stainless steel surfaces, and equivalent when testing unprocessed corn-based products, it is not validated for the same variety of matrices as the Bia Diagnostics Gluten LFD. Additionally, when testing processed corn based products it calls for an extremely long (100 minute) extraction time. Food manufacturers must decide based on their needs which method to use in their management program.

3.5 Conclusions
The Bia Diagnostics Gluten LFD is a fast and reliable method of qualitatively detecting gluten down to 5 ppm in buckwheat, chocolate syrup, soy milk, corn flakes, clean in place solutions, and on stainless steel surfaces. It meets the requirements of the AOAC PTM program for screening these foods for gluten and may be used to show regulatory compliance as part of a management program in gluten-free production. At levels below 5 ppm results become less consistent, depending on matrix and the amount of gluten present. As all food matrixes are different, the method should be validated for a specific matrix to determine its limit of detection in that application.
REFERENCES


Ferguson, A., Arranz, E., & O'Mahony, S. (1993). Clinical and pathological spectrum of coeliac disease--active, silent, latent, potential. *Gut, 34*(2), 150-151. [https://doi.org/10.1136/gut.34.2.150](https://doi.org/10.1136/gut.34.2.150).


New Zealand (2008). Australia New Zealand Food Standards Code, Standard 1.2.8: Nutrition Information Requirements


Appendixes

Appendix 1. 20 ppm Incurred Brownie Recipe

Ingredients

- 415g sugar
- 156g unsalted butter
- 6g salt
- 6g gluten-free vanilla extract
- 95g Dutch-process cocoa or baking cocoa
- 207g liquid egg
- 156g Bob’s Red Mill Gluten-Free Multi-Purpose Flour
- 6g baking powder

Instructions

1. Preheat KitchenAid Countertop Oven KC0223 (KitchenAid, Benton Harbor, MI) to 350°F. Grease one 2" deep pan.
2. Place the sugar, butter, and salt in a microwave-safe bowl. Microwave until the butter melts and the mixture lightens in color, stirring occasionally.
3. Blend in the vanilla and cocoa, then add the eggs and mix until shiny.
4. Blend in the flour or flour blend and the baking powder, mixing well.
5. Separate a 200g portion of batter into a clean mixing bowl. To this portion, add 4ml of 1000 ppm gluten spike in 60% ethanol, for a 20 ppm spiked sample.
6. Mix the spiked sample well
7. Pour the spiked batter into the prepared pan, spreading it to the edges.
8. Bake the brownies for 35 minutes.
9. Remove from the oven and let cool completely before cutting. Freeze immediately.
Appendix 2. 20 ppm Incurred Muffin Recipe

Ingredients

- 9g baking powder
- 1g baking soda
- 3g xanthan gum
- 3g salt
- 262g Bob’s Red Mill Gluten-Free Multi-Purpose Flour
- 113g soft butter
- 131g sugar
- 43g molasses or honey
- 100g liquid egg
- 335g mashed very ripe bananas (about 2 medium bananas)

Instructions

1. Preheat KitchenAid Countertop Oven KC0223 (KitchenAid, Benton Harbor, MI) to 375°F. Grease a 12-cup muffin pan, and line with papers.
2. Mix together the baking powder, baking soda, xanthan gum, salt, cinnamon, and flour blend. Set aside.
3. Beat together the soft butter, sugar, and molasses.
4. Beat in the eggs one at a time, scraping the bottom and sides of the bowl between additions.
5. Add the dry ingredients to the mixture in the bowl in three parts, alternately with the mashed bananas, beating well between additions and scraping the bottom and sides of the bowl one or two times during this process. After everything is added, scrape the bowl again and beat at medium high speed for 30 seconds.
6. Separate a 200g portion of the batter into a clean mixing bowl. To this portion, add 4ml of 1000 ppm gluten spike in 60% ethanol, for a 20 ppm spiked sample.
7. Mix the spiked sample well.
8. Scoop the batter into the prepared pan, filling the cups to the top.
9. Bake the muffins for 25 minutes. Remove from the oven and let cool completely. Freeze immediately.
Appendix 3. 20 ppm Inocreed Cookie Recipe

Ingredients

- 210g Bob’s Red Mill Gluten-Free Multi-Purpose Flour
- 3g baking soda
- 2g kosher salt
- 170g semi-sweet chocolate chips
- 100g granulated sugar
- 218g packed light brown sugar
- 250g certified gluten free old fashioned rolled oats
- 140g unsalted butter, at room temperature
- 120g liquid egg beaters
- 4g pure vanilla extract

Instructions

1. Preheat KitchenAid Countertop Oven KC0223 (KitchenAid, Benton Harbor, MI) to 350°F. Line a rimmed baking sheet with parchment paper and set it aside.

2. In a large bowl, place the flour, baking soda and salt, and whisk to combine well. Place the chocolate chips in a medium-sized bowl, add one tablespoon of the whisked dry ingredients, and toss the chips to coat them evenly. Set the chips aside. Add the granulated and light brown sugars to the large bowl of dry ingredients, and whisk again to combine well, breaking up any lumps in the brown sugar. Add the oats and stir to combine well. Add the butter and mix until combined. Add the beaten eggs and vanilla, and mix until the dough comes together. Add the chocolate chips and reserved dry ingredients to the dough, and mix to distribute the chips evenly throughout.

3. Separate a 200g portion of the batter into a clean mixing bowl. Add 4mls of 1000 ppm gluten spike in 60% ethanol, for a 20 ppm spiked sample.

4. Mix the spiked sample well

5. Divide the dough into equal pieces, each about the size of a golf ball, on the prepared baking sheet, about 2 inches apart. Place the baking sheet in the freezer until firm, about 15 minutes. Once chilled, place the baking sheet in the center of the preheated oven, and bake for 20 minutes. Remove the baking sheet from the oven and let cool completely. Freeze immediately.
Appendix 4. 0 ppm and 20 ppm Incurred Bread Recipes

Ingredients

- 126g sorghum flour
- 190g potato starch
- 64g tapioca flour
- 16g baking powder
- 8g salt
- 6g xanthan gum
- 6g guar gum
- 16ml olive oil
- 90ml H20

Instructions

1. Preheat KitchenAid Countertop Oven KC0223 (KitchenAid, Benton Harbor, MI) to 400°F. Line a baking tray with parchment paper and set it aside.
2. In a large bowl, mix dry ingredients together well. Mix in liquid ingredients slowly.
3. Separate a 200g portion of the batter into a clean mixing bowl. Add 4mls 60% ethanol, for a 0 ppm spiked sample.
4. Separate a separate 200g portion of the batter into a clean mixing bowl. Add 4mls of 1000 ppm gluten spike in 60% ethanol, for a 20 ppm spiked sample.
5. Mix both samples well
6. Shape each dough into a small football sized loaf. Place on baking sheet and bake for 25 minutes. Remove from oven and let cool completely. Freeze both loaves immediately.