



Development of a barcode-style lateral flow immunoassay for the rapid semi-quantification of gliadin in foods



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ABSTRACT

In this work, a barcode-style lateral flow immunoassay is developed using two cut-off values (10 and 50 mg kg⁻¹ gliadin) to provide a semi-quantification for identifying “gluten-free” and “very low gluten” foods, based on the international Codex Alimentarius Standard. This developed assay exhibits favorable specificity in differentiating wheat from seven commonly used grains, with only a slight cross-reaction with barely. The intra-assay and inter-assay CV values of this assay were 1.5–1.7% and 2.5–4.5%, respectively, revealing high reproducibility. In the analysis of 48 food samples, the results of this assay closely agreed with those obtained using AOAC-approved ELISA or strip kits, as the Cohen's kappa coefficients for both comparisons exceeded 0.8. Thus, this developed assay can be used to quickly estimate the gliadin content in foods in order to protect people with wheat allergy or celiac disease from the accidental ingestion of gliadin.

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1. Introduction

Gliadin in wheat is an alcohol-soluble compound that contains approximately 50% of the wheat gluten protein and it is also a food allergen. After a patient with wheat allergy accidentally ingest food containing gliadin, the gliadin allergen crosses the intestinal barrier and binds to the IgE molecules tagged on the surface of mast cells, thereby triggering degranulation inside mast cells to release different inflammatory cytokines that can induce various allergic reactions (Sampson, 1999). In early childhood, the main symptoms of wheat allergy are nausea, abdominal pain, urticaria, angioedema, bronchial obstruction, even systemic anaphylaxis, and delayed symptoms include gastrointestinal symptoms and atopic dermatitis (Kotaniemi-Syrjänen et al., 2010). The adult symptoms of wheat allergy include wheat-dependent exercise-induced anaphylaxis and wheat-induced anaphylaxis (Park et al., 2011). According to previously investigations, the prevalence of wheat

allergy is 0.21% in Japanese adults, 5.7% in Turkish children, and 6.1% in Swiss children (Ferrari & Eng, 2011; Morita et al., 2012; Yavuz et al., 2010). As well as triggering wheat allergy, gliadin also induces celiac disease (CD), which involves inflammation of the small-intestinal mucosa and affects 1 in 133 Americans (Fasano et al., 2003). Nowadays, patients can only follow a lifelong gluten-free diet to improve symptoms and thus many developed countries have established a food labeling regulation specific for gluten, in order to protect patients from the accidental ingestion of gluten. Based on the revised International Codex Alimentarius Standard published in 2008, a food with a gluten level that does not exceed 20 mg kg⁻¹ (<20 mg kg⁻¹) can be labeled as “gluten-free” and a food with a gluten level of between 20 and 100 mg kg⁻¹ (20–100 mg kg⁻¹) can be labeled as “very low gluten”.

The analysis of residual gliadin levels in foods is often quite challenging, because of the complexity of the food matrices. Therefore, various methods for quantifying gliadin in food have been developed. Nassef et al. (2008) described an electrochemical immunosensor for determining the amount of gliadin in foods, and the sensitivity of this analytical method was 5.5 ng mL⁻¹ gliadin. Liquid chromatography coupled with tandem mass

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spectrometry (LC–MS/MS) can be used to identify gluten compounds at very low levels in a food matrix, with a detection range of 1–30 pg mg⁻¹ (Sealey-Voyksner, Khosla, Voyksner, & Jorgenson, 2010). A combined method that incorporates anti-gliadin antibody-conjugated immunomagnetic beads (IMBs) to capture gliadin in samples and fluorescent dye-loaded immunoliposomal nanovesicles (IMLNs) to produce and enhance a detection signal has also been successful in detecting gliadin, and it has a favorable sensitivity as low as 0.6 µg mL⁻¹ gliadin (Chu & Wen, 2013). Currently, enzyme-linked immunosorbent assays (ELISAs) are one of the favored approaches for detecting residual gluten in foods, including sandwich and competitive assays (Sunwoo, Gujral, Lutz, & Suresh, 2012; Thompson & Méndez, 2008; Valdés, García, Llorente, & Méndez, 2003). Their detection limits are mostly in the low ppm range. In addition, some ELISAs utilize the R5 monoclonal antibody, which specifically reacts with the celiac toxic epitope QQPFP of gliadin, in order to improve assay specificity. ELISAs are reliable and accurate, but they must be carried out over long period; involve multiple steps, and depend on non-portable expensive devices. To eliminate these problems, this work develops a fast, accurate and reliable lateral flow immunoassay (LFA) without any expensive reading device for the rapid on-site detection of gliadin.

The LFA is a membrane-based immunochromatographic method for use in the field or at home. Typically, this qualitative test is suitable for analyzing the target at a concentration up to a known limit. For example, the most popular application of LFAs is to detect human chorionic gonadotropin during pregnancy, and the detection result can be easily viewed by the naked eye after about 10 min of reaction without expensive laboratory equipment. Recently, to reduce the time and cost of analysis, various multiplex LFAs have been developed by adding more target test lines to the membrane to enable various targets to be identified simultaneously. For instance, multiplex LFAs have been applied to identify two mycotoxins (deoxynivalenol and zearalenone) in food and feed (Kolossova, De Saeger, Sibanda, Verheijen, & Van Peteghem, 2007), or to identify three serogroups of *Escherichia coli* O157, O26, and O111 with the detection limits in 25 g of ground beef as 6.3, 2.9, and 5.6 CFU, after 18 h of enrichment (Yonekita et al., 2013). Normally, the results of an LFA are yes/no, in the form of the absence of the test line on strips by visual observation. Therefore, obtaining a quantitative data from an LFA typically requires an image analyzer or a comparison with a color scale card. To eliminate this need of using an additional device, barcode-style LFAs have been developed to provide rapidly a semi-quantitative result, based on the relationship between the number of test lines appearing on the strip and the analyte concentration. Due to its simplicity, the barcode-style LFA has been utilized in several studies for estimating the concentration of various target analytes, such as lipoprotein A in plasma (Lou, Patel, Ching, & Gordon, 1993), C-reactive protein in whole blood (Leung et al., 2008), hydrogen peroxide (Fung, Chan, & Renneberg, 2009), and prostate acid phosphatase in serum (Fang, Chen, Li, & Xia, 2011).

In this work, for the semi-quantification of gliadin levels in foods, a barcode-style LFA was developed in which immuno-gold nanoparticles (immuno-AuNPs) serve as the detection reagent, on which chicken polyclonal anti-gliadin IgY antibodies are non-covalently conjugated. When the strip of this developed assay is dipped into the test solution, “gliadin-immuno-AuNP complexes” are formed and flow along the strip, as described as Scheme 1. If no gliadin is present in a sample (a negative test), the immuno-AuNPs are captured by anti-chicken antibodies dispensed at the control line, producing one obvious red control line. If a sample contains gliadin, then the immuno-AuNPs reacts with gliadin to form “gliadin-immuno-AuNP complexes”, which are subsequently captured by the anti-gliadin antibodies immobilized

on the test lines. The design with two test lines is the crucial development in this work, as it provides the semi-quantitative detection of gliadin. The cut-off value for test line 1 was set to 10 mg kg⁻¹ gliadin and that for test line 2 was set to 50 mg kg⁻¹ gliadin, to conform to the Codex recommendation for the labeling of “gluten-free” and “very low gluten” foods. In the assay, as the gliadin concentration increases, the analyte causes the test lines to appear in a graduated sequence, such that the appearance of each test line indicates that a cut-off concentration of gliadin has been reached. The results obtained using this developed assay were compared with those obtained using the Association of Official Analytical Chemists (AOAC)-approved gliadin-ELISA kit and EZ Gluten test kit to validate its precision and feasibility.

2. Materials and methods

2.1. Materials

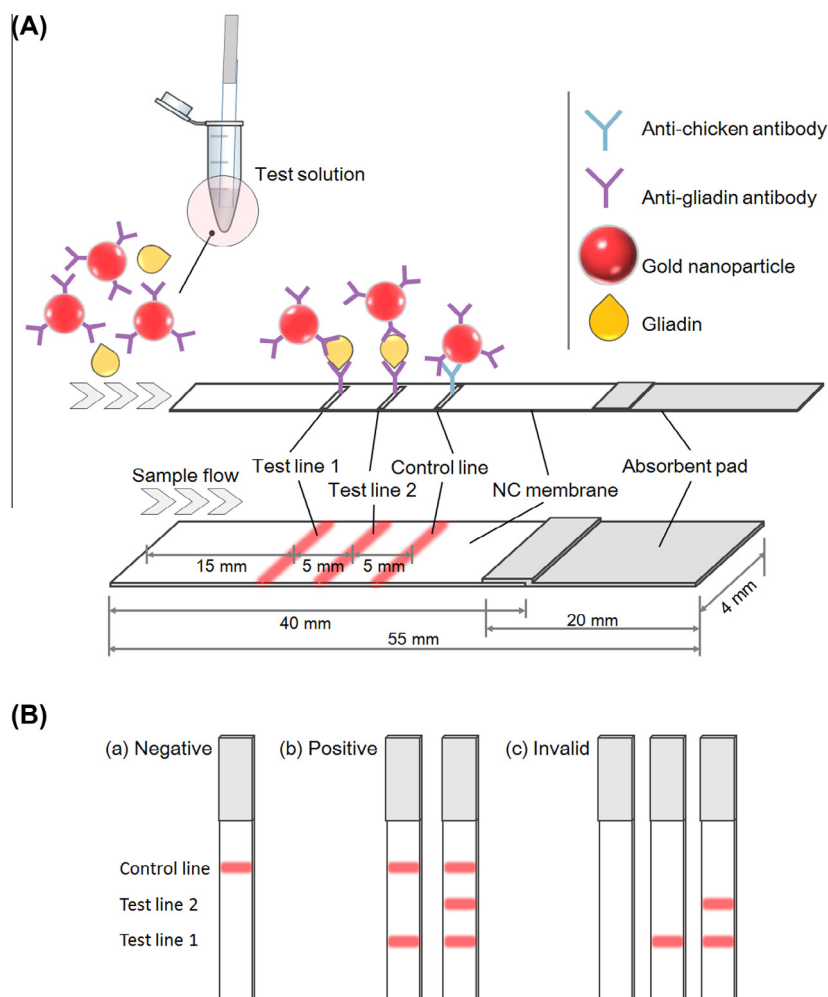
Nine crops (wheat, barley, oat, rice, foxtail millet, corn, buckwheat, soybean, and rye) were purchased from local supermarkets (Taichung, Taiwan). The goat anti-chicken IgY antibody was obtained from Jackson ImmunoResearch (PA, USA). Gliadin, bovine serum albumin (BSA), polyvinylpyrrolidone (PVP), hydrogen tetrachloroaurate trihydrate (HAuCl₄), and sodium citrate were bought from Sigma–Aldrich Co. (MO, USA). Absorbent pads and Hi-Flow Plus nitrocellulose (NC) membranes were purchased from Merck Millipore (MA, USA). Two AOAC-approved kits, the RIDASCREEN® Gliadin kit (R-Biopharm, Germany) and the EZ Gluten test kit (ELISA Technology, USA), were utilized as the standard kits for estimating the concentration of gliadin in foods.

2.2. Preparation of gold nanoparticles (AuNPs)

AuNPs were prepared by the reduction of gold chloride with sodium citrate according to a reported method (Saraiva & de Oliveira, 2002). Briefly, 10 mL of 0.1% gold chloride trihydrate solution was heated with 15 mL of deionized water in a Erlenmeyer flask to 120 °C with vigorous stirring. To generate the 20 nm AuNPs, 2.4 mL of 38.8 mM sodium citrate were added to the boiling solution with stirring. When the solution changed from light yellow to wine-red, the reaction mixture was boiled for another 10 min to complete the reduction of the gold ions. The size of the AuNPs was measured by a dynamic light-scattering method using a nanosizer 90ZS (Malvern Instruments, Worcestershire, UK). The AuNP solution was stored at 4 °C and was used to prepare immuno-AuNPs.

2.3. Preparation of anti-gliadin antibody-tagged AuNPs (immuno-AuNPs)

The immuno-AuNP solution in this work was prepared by mixing 2.5 mL of 6.3 mM potassium carbonate solution and 7.5 mL of the AuNP solution. After vortex mixing, 1 µg mL⁻¹ of anti-gliadin antibody (2.5 mL) was added to the pH-adjusted 20 nm AuNP solutions. The methods to produce and purify anti-gliadin IgY was mentioned in our previous study (Chu, Lin, Chen, Chen, & Wen, 2012). Briefly, 40-week-old white Leghorn hens were immunized using gliadin-complete Freund's adjuvant mixture and then boosted using gliadin-incomplete Freund's adjuvant. After the third boost, eggs were collected daily and anti-gliadin antibody was purified from the egg yolk using the PEG-alcohol method. To block the uncoated sites of immuno-AuNPs, 10% of BSA was added and incubated at room temperature for 1 h. Finally, the blocked immuno-AuNP solutions were centrifuged at 10,000 rpm at 4 °C



Scheme 1. Barcode-style lateral flow immunoassay for detecting gliadin. (A) The strip consists of two components: an absorbent pad and a nitrocellulose (NC) membrane. The two important regions on the NC membrane are the control line and the test zone (test line 1 at the bottom and test line 2 at the top). The control line is coated with goat anti-chicken antibody and the test lines are coated with chicken anti-gliadin antibody. (B) Results: (a) a negative test (absence of gliadin): immuno-AuNPs are captured on the control line and a single red line is produced; (b) positive tests: immuno-AuNPs migrate to the control line and gliadin-immuno-AuNP complexes are captured on the test zone. One or two red test lines are generated, reflecting the concentration of gliadin (cut-off value of test line 1 is 10 mg kg^{-1} gliadin, and that of test line 2 is 50 mg kg^{-1} gliadin), and (c) invalid tests: if no band appears on the control line, the test is invalid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for 20 min and the pellets were suspended in 10 mM phosphate buffer (pH 7.4) and stored at 4 °C until use.

2.4. Assembly of strips of barcode-style lateral flow immunoassay

First, goat anti-chicken IgY antibody and anti-gliadin IgY antibody were immobilized onto the NC membrane as the control line and test lines using a LINOMAT V automatic sample spotter (CAMAG, Muttenz, Switzerland), respectively. The distance between each line was 5 mm. The coating density of antibodies on the control line was $3 \mu\text{g cm}^{-1}$ and that of each test line was $30 \mu\text{g cm}^{-1}$. The spray rate for coating antibody on the NC membrane was 70 nL s^{-1} at a relative humidity of 50%. After coating, the membrane was dried in air and then treated with a blocking buffer (0.02 M Tris-base, pH 7, containing 0.1% PVP and 0.5% BSA) for 30 min at room temperature. Following the drying of the membrane in an oven at 30 °C for 1 h, an absorbent pad (4 mm × 20 mm) was glued to on one end of an NC membrane (4 mm × 40 mm) with an overlap of 5 mm. The strips thus formed were stored in a sealed tube under dry conditions at room temperature.

2.5. Pretreatment of sample

Nine crops (wheat, barley, oat, rice, foxtail millet, corn, buckwheat, soybean, and rye) and various commercial food samples were crushed in a mill to produce fine flour. The method of extraction of gliadin that is used herein resembles that used in the work of van den Broeck (Van den Broeck et al., 2009). Initially, water-soluble proteins in a 1 g food sample were removed by shaking at 50 rpm in deionized water at room temperature for 1 h. After the water-soluble proteins had been extracted twice, globulins were extracted from the flour residue using 0.5 M NaCl solution. Finally, the pellet was extracted with 10 mL of 60% (v/v) ethanol. The extract was centrifuged at 8000 rpm for 10 min, and the supernatant was filtrated using a filter with 0.45 μm pores for use as the gliadin sample that was subsequently analyzed using the developed barcode-style lateral flow immunoassay and two AOAC-approved commercial kits.

2.6. Assay procedure

In this work, immuno-AuNPs (30 μL) were the detection reagent, and were incubated with each sample (10 μL) at room

temperature for 30 min to react with gliadin molecules in the samples. After incubation for 30 min, a strip was dipped into the test solution, which was absorbed into and migrated up a strip. The color intensities of the test lines of strips were either visually estimated by the naked eye or measured using “CHR-120R (G) v0.9.0” software in a CHR-120R Chromogenic Reader (Kaiwood Technology Co., Taiwan). The reader was calibrated before measurement, and the color intensity of the line on a strip was expressed as relative light units (RLU).

2.7. Sensitivity and specificity of developed assay

Sigma gliadin ($0\text{--}100\text{ }\mu\text{g mL}^{-1}$) was used as the standard and freshly prepared in 60% (v/v) ethanol. To obtain quantitative results, the color intensity of each test line of each strip was measured using a Chromogenic Reader. To evaluate the specificity of this developed assay, extracts of nine crops (wheat, barley, oat, rice, foxtail millet, corn, buckwheat, soybean, and rye) were prepared and analyzed using the developed assay.

2.8. Statistical analysis

All data that were obtained using the developed barcode-style LFA and the AOAC approved kits were presented as mean $\pm$ SD. All results were statistically analyzed using the SAS statistical package (SAS Institute, NC, USA). A *p*-value of less than 0.05 was regarded as indicating significance.

3. Results and discussion

3.1. Production and optimization of immuno-AuNPs

To optimize the binding between antibodies and AuNPs, the effects of using AuNP solutions at various pH values and antibody concentrations were evaluated (Roth, 1982). In this work, two batches of immuno-AuNPs with different diameters (20 and 45 nm) were used to investigate the effect of their size on the assay performance. The stability of AuNPs in a solution depends on the electrostatic repulsion among particles, so the presence of ionic substances (such as NaCl) can cause AuNP aggregation (Zhou, Ralston, Sedev, & Beattie, 2009). Under such conditions, instability may be prevented by coating the AuNP surfaces with proteins to provide steric hindrance. Since the immobilization of antibody on an AuNP surface is based on the electrostatic effect, the pH of an AuNP solution is generally adjusted before antibodies are added, and the amount of a particular protein that is adsorbed on an AuNP surface reaches a maximum at its isoelectric point (pI), or 0.5 pH units higher than the pI (Paek, Lee, Cho, & Kim, 2000). Hence, in this work, potassium carbonate was prepared in various concentrations to adjust the pH, and the OD₆₂₅ value was utilized to indicate the stability of the AuNPs. A higher OD₆₂₅ value represents greater aggregation of AuNPs (Zhang, Leem, Srisombat, & Lee, 2008). The results reveal that as the potassium carbonate concentration is increased, the OD₆₂₅ value of the AuNP solution increased and rose markedly as the concentration of potassium carbonate increased beyond 6.3 mM (data not shown). Therefore, potassium carbonate solutions with concentrations lower than 6.3 mM were selected to optimize the conjugation of antibodies on the AuNPs. As displayed in Fig. S1, the intensity of the OD₆₂₅ signal decreased as the potassium carbonate concentration increased, and 6.3 mM yielded the lowest OD₆₂₅ value for both batches of AuNPs. When they were treated with a higher concentration of potassium carbonate, the AuNPs were stabilized by a lower antibody concentration, perhaps because a higher concentration of potassium carbonate generated a higher charge density on AuNP surface,

causing the AuNPs to repel each other more strongly, so fewer antibodies were required to provide steric hindrance to prevent aggregation of immuno-AuNPs. To reduce the cost of the process, the lowest antibody concentration that ensured the stability of AuNPs with the lowest obtained value of OD₆₂₅ was regarded as the optimal concentration for preparing immuno-AuNPs. The optimal concentrations of antibodies for treating 20 nm and 45 nm AuNPs were 1 mg mL^{-1} and 2 mg mL^{-1} , respectively, as shown in Fig. S1. The 45 nm AuNPs required more antibodies possibly because they got a larger total surface area needed to be covered by antibodies than the 20 nm AuNPs did.

3.2. Optimization of barcode-style lateral flow immunoassay

To optimize the assay performance, factors such as the diameter of the AuNPs, the immuno-AuNP/sample volume ratio, the type of the NC membrane, and the antibody density on the test line 1 were optimized as follows. First, the size effect of AuNPs on the signal intensity on a strip was evaluated using two batches of immuno-AuNPs with different diameters (20 nm and 45 nm). Fig. 1(A) presents the visual appearance of the strips and the total color intensity of the two test lines in the assays using the differently sized AuNPs for detecting various concentrations of gliadin samples. The total color intensity of the two test lines in the assay with 20 nm AuNPs to detect $50\text{ }\mu\text{g mL}^{-1}$ gliadin was 1.3 times that of those with 45 nm AuNPs. The 20 nm and 45 nm AuNPs resulted in bright red and purple analysis lines, respectively. A purple detection line on a strip commonly made distinguishing the result by the naked eye difficult due to the decrease of the intensities of the lines (Mao et al., 2009). In addition, the strips with the 45 nm AuNPs had a darker background possibly due to the aggregation of AuNPs. Previous investigations have found that AuNPs with a large diameter ($>40\text{ nm}$) are unstable and they easily self-coagulated (Fang et al., 2011). Therefore, 20 nm AuNPs were utilized in the following experiments. Moreover, since the AuNPs/sample volume ratio directly affects the signal intensity of the test lines, various ratios were tested. As presented in Fig. 1(B), the total color intensity in detecting gliadin greatly increased as the volume ratio rose from 1:3 to 3:1 ($24.47 \pm 1.46\text{ RLU}$ to $82.46 \pm 3.66\text{ RLU}$). This intensification of the assay signal may have occurred because an assay with a larger AuNPs/sample volume ratio can provide more immuno-AuNPs to capture gliadin molecules in samples, resulting in a stronger signal on the test lines of the strips. Since the assay with the volume ratio of 3:1 yielded the strongest signal in detecting gliadin, this ratio was used throughout the rest of this work.

In an immunochromatographic strip assay, the flow rate of a sample through the membrane determines the signal intensity on the test lines (Liu et al., 2009). According to the manufacturer of the NC membranes, the “catalog number” is defined as the time taken for a sample front to flow through a distance of 4 cm (seconds per 4 cm) on a membrane. In this work, four NC membranes – HF075, HF120, HF135 and HF240 – were utilized to study the effect of the flow rate on the signal intensity on the strips. Fig. 1(C) shows the visual appearance and the total color intensity of the test lines in the detection of gliadin with four NC membranes. The average width of the test lines on the HF075, HF120, HF135 and HF240 membranes were 3.30 mm, 2.45 mm, 1.10 mm and 0.85 mm, respectively. Among four membranes, HF135 membrane had the best analytical lines, with a visually clear color, the highest color intensity, and the optimal width. Relative to those of HF075 and HF120, the low flow rate of HF135 led to a longer time for gliadin molecules in a sample to react with the antibodies tagged on the AuNPs or immobilized on the NC membrane. Low et al. indicated that the flow rate of a sample solution through the membrane and the affinity of antibodies to targets influence the sharpness and width of test lines on the membrane (Low et al., 2013). Additionally, in the present work,

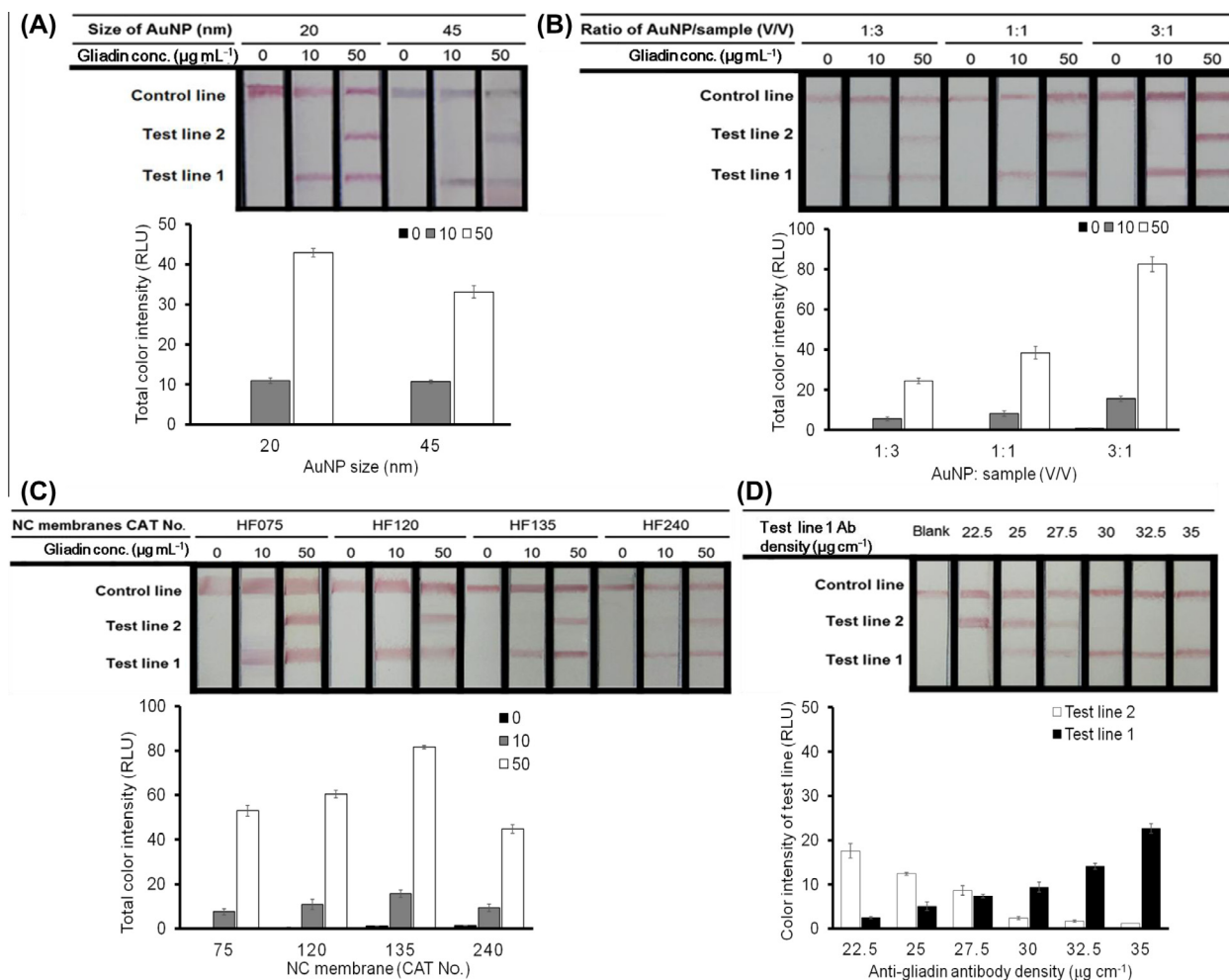


Fig. 1. Optimization of parameters of operation of the developed lateral flow immunoassay for detecting gliadin: (A) the particle diameter of immuno-AuNPs; (B) the AuNP/sample volume ratio (v/v) with a final volume as 40 μL ; (C) the type of the NC membrane; and (D) the coating density of anti-gliadin antibody on test line 1 of a strip.

a dark red band was observed at the end of membrane HF240, where the membrane reacted with the sample solution. This dark band revealed the aggregation of AuNPs, perhaps owing to the relatively small size of the pores of the HF240 membrane, and their consequent interference with AuNP migration along the membrane such that the signal intensities on the test lines and control line were weakened. Thus, the HF135 membrane was chosen in subsequent studies. Finally, to distinguish easily between “gluten-free” foods ($<10 \mu\text{g mL}^{-1}$ of gliadin) and “very low gluten” foods ($10\text{--}50 \mu\text{g mL}^{-1}$ of gliadin) using the number of visible test line on a strip, the coating density of anti-gliadin antibodies on test line 1 was quantified. As displayed in Fig. 1(D), the color intensity of test line 2 gradually declined while that of test line 1 increased as the antibody density on test line 1 increased. More antibodies on test line 1 captured more “gliadin-immuno-AuNP complexes” so a smaller proportion of these complexes remained to be captured on test line 2. The color intensity on test line 2 was lower than the visual detection limit of 4 RLU when the antibody density on test line 1 had increased to $30 \mu\text{g cm}^{-1}$ in the detection of $10 \mu\text{g mL}^{-1}$ gliadin. Accordingly, $30 \mu\text{g cm}^{-1}$ was used as the optimal antibody density on test line 1.

3.3. Sensitivity and specificity of the developed assay

To evaluate the working range and cut-off values of the developed assay, various concentrations of gliadin were analyzed by this

assay. Fig. 2(A) presents images of various strips in detecting gliadin in 60% ethanol or in pancake mixture. Test line 1 first turned red when the gliadin concentration was $10 \mu\text{g mL}^{-1}$; therefore, the cut-off concentration of gliadin of test line 1 was determined to be $10 \mu\text{g mL}^{-1}$ in the buffer and food samples. When low concentrations of gliadin ($10\text{--}40 \mu\text{g mL}^{-1}$) were detected, only one red band appeared on test line 1 of each strip. Test line 2 first presented a second visible red band when the sample had a gliadin concentration reached $50 \mu\text{g mL}^{-1}$, and two red test lines were clearly observed at higher concentrations of gliadin ($50\text{--}100 \mu\text{g mL}^{-1}$). Hence, the cut-off value for test line 2 was determined to be $50 \mu\text{g mL}^{-1}$. Hence, this developed assay is very useful to identify “gluten-free” ($<10 \mu\text{g mL}^{-1}$ gliadin; no test line appears), “very low gluten content” (≥ 10 to $< 50 \mu\text{g mL}^{-1}$ gliadin; only test line 1 is red), and “high gluten content” ($\geq 50 \mu\text{g mL}^{-1}$ gliadin; two red test lines appear), because its cut-off values both match the Codex recommendations for food labeling of “gluten-free” and “very low gluten” foods. Moreover, the total color intensities of test lines 1 and 2 were measured to plot a dose–response curve of total color intensity as a function of gliadin concentration. The dose–response curves in 60% ethanol and in a food sample were linear from 8 to $90 \mu\text{g mL}^{-1}$ gliadin, and the R^2 values in 60% ethanol and in a food sample were 0.986 and 0.987, respectively. In previous studies, gliadin has been detected using various assays, such as a double antibody sandwich ELISA with a detection limit of 5 ng mL^{-1} gliadin (Sunwoo et al., 2012)

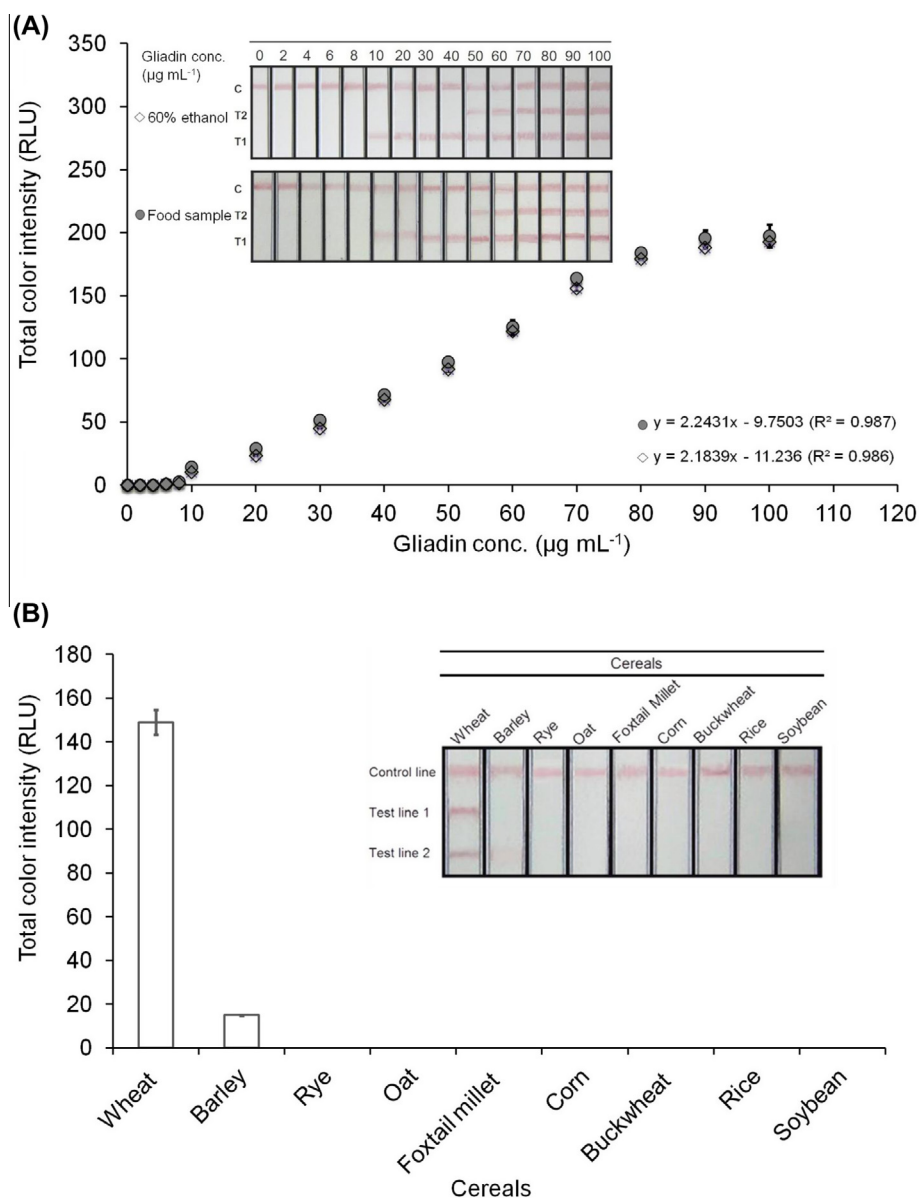


Fig. 2. Images of strips and dose–response curves of total color intensity against gliadin concentration for detecting gliadin at concentrations from 0 to 100 $\mu\text{g mL}^{-1}$ in 60% ethanol and in food sample (A), and the specificity of the developed assay: images of strips and total color intensity for detecting alcohol-soluble proteins in nine common cereals (B).

or a real-time PCR with a sensitivity as low as 100 ng mL^{-1} (Pinto et al., 2014). Although the developed assay was less sensitive than the above assays, it is a more convenient and rapid system for on-site analysis. To the best of the authors' knowledge, the assay established in this study is the first semi-quantitative LFA that has been developed for detecting gliadin in foods.

Before being utilized to detect gliadin in foods, the specificity of the developed assay is testified by analyzing nine commonly used cereals. As shown in Fig. 2(B), only the wheat sample produced two clear red test lines, indicating the specificity of the developed assay. No other cereal yielded any positive results, except for barley which yielded only a weak positive result, with a slightly red band on test line 1, perhaps because both wheat and barley are members of the Poaceae family, and so their proteins may share structurally similar epitopes, possibly causing the polyclonal anti-gliadin antibody bind non-specifically to barley (Liebers et al., 1996). In addition, several studies have found that hordein in barley cross-reacts with gliadin in wheat, which may be due

to that gliadin and hordein are rich in proline and glutamine and both are belonged to the group of prolamins (Palosuo et al., 2001; Sánchez et al., 2007). Since no band was observed on the strips that were utilized with the other seven cereals, the developed assay exhibited no cross-reactivity with the other seven cereals and so accurately detect wheat allergens in foods with high specificity.

3.4. Reproducibility

Evaluating the repeatability of the developed dipstick assay is important to determine its consistency of performance. Hence, samples of a gliadin-free pancake mix that was spiked with gliadin at two concentrations were analyzed using the newly developed assay to estimate the intra-assay precision by assessing ten repeats of each sample panel in one analytical run. The inter-assay precision was determined by performing two analyses per day for five consecutive days, and using different lots of barcode-style strips

on different days. The coefficient of variation (CV) was calculated as the ratio of standard deviation (SD)/mean $\times 100$. Table 1 presents the visual appearances of the strips and the calculated parameters of assay precision. The calculated intra-assay CV value was 1.5–1.7%, while the inter-assay CV value was 2.5–4.5%, respectively. The precision of this developed assay was satisfactory because the intra-assay and inter-assay CV values were both less than 15% (Venosa, King, & Sorial, 2002).

3.5. Analysis of commercial food products

The feasibility of using this novel assay on processed foods was investigated by detecting gliadin in 48 commercial food products. The results obtained using the assay were compared with those obtained using two AOAC-approved kits – the RIDASCREEN Gliadin ELISA kit and the EZ Gluten test strip kit – as shown in Table 2. The AOAC-approved ELISA kit identified 24 samples as “gluten-free” foods ($<10 \text{ mg kg}^{-1}$ gliadin), 18 samples as “very low gluten” foods ($10\text{--}50 \text{ mg kg}^{-1}$ gliadin), and six samples as gluten-containing food samples ($>50 \text{ mg kg}^{-1}$ gliadin). The identification of “gluten-free” foods was consistent with all of their official labels, “Controlado por FACE”, which is guaranteed by the Spanish Federation of Celiac Associations. Moreover, among the 24 “gluten-free” food samples identified by the ELISA kit, three yielded weakly positive results when tested using the developed assay, perhaps because these food samples contained some interfering substances that bound non-specifically to antibodies of the developed assay. Six out of the 24 “gluten-free” food samples yielded positive results when tested using the EZ Gluten test strip kit. The detection limit of this EZ Gluten test strip kit was 10 mg kg^{-1} gluten (approximately 5 mg kg^{-1} gliadin) and the gliadin levels in these six samples all exceeded 5 mg kg^{-1} , according to the results of the ELISA kit. Hence, the positive results from these samples that were obtained using the EZ Gluten test strip kit were reasonable. Furthermore, the results for the 18 “very low gluten” foods and the six “gluten containing” foods, as identified by ELISA kit all matched the results of the developed assay.

Furthermore, the reliability parameters of the developed assay were as provided, based on using the AOAC-approved RIDASCREEN Gliadin ELISA kit as the standard kit (Table 3). In the analysis of 24 “gluten-free” foods, the sensitivity was 0.88 (21/24), while, the specificity was 1.0 (24/24). In the analysis of 18 “very low gluten” foods, the sensitivity was 1.0 (18/18), but the specificity was slightly reduced to 0.9 (27/30) (Akobeng, 2007). In the analysis of six “gluten-containing” foods, the developed assay performed very well with both sensitivity and specificity of 1. Moreover, Cohen's kappa test was conducted to calculate the degree of agreement between the results obtained using the above two assays (Cohen, 1968). The kappa coefficients (κ) for the identification of products with different gliadin ranges all exceeded 0.8, revealing “almost perfect” concordance for these two assays (Landis & Koch, 1977). Therefore, the performance of this developed assay was comparable with that of the AOAC-approved ELISA kit in analyzing commercial food products. Although ELISA is a sensitive and reliable method, it requires expensive equipment and trained personnel. In contrast, this developed barcode-style LFA involves no detector and is user-friendly; therefore, it is better suited to routine on-site use. In addition, the solubility of gliadin in processed food may be significantly reduced and this influence can be depended on the conditions of thermal processing and the compositions of foods. Hence, it is possible to underestimate the level of gliadin in processed foods, when using the assays that need the extraction of gliadin for sample preparation.

3.6. Estimation of cost

Lateral flow assays use a variety of labels, such as gold, silver or silica particles, or latex beads. AuNPs are the most popular label because of their stability, low cost per assay and ease conjugation with antibodies (Dzantiev, Byzova, Urusov, & Zherdev, 2014). Thus, AuNPs were utilized as the detection reagent in the developed assay. Zhou, Mao, and Juncker (2012) developed an immunochromatographic assay on a thread (ICAT) and the cost per test was estimated

Table 1
Visual appearances of tests to determine intra-assay precision (A) and inter-assay precision (B) of the developed assay in analyzing samples with different concentrations of spiked gliadin (0, 10, and $50 \mu\text{g mL}^{-1}$), and their relevant calculated results (C).

(A)

(B)

(C)

experiment	spiked gliadin concentration ($\mu\text{g mL}^{-1}$)	n	measured gliadin concentration (mean $\pm$ SD; $\mu\text{g mL}^{-1}$)	recovery (%)	CV (%)
Intra-assay	10	10	10.8 ± 0.2	107.9	1.7
	50	10	47.4 ± 0.7	94.8	1.5
Inter-assay	10	10	11.2 ± 0.5	111.6	4.5
	50	10	46.4 ± 1.2	92.8	2.5

Table 2

Comparison of barcode-style lateral flow immunoassay, and two AOAC-approved kits (EZ Gluten test strip kit and RIDASCREEN gliadin ELISA kit) for detection of gliadin in different food samples ($n = 3$).

Assays	Barcode-style immunochromatographic strip assay	EZ Gluten test strip kit	RIDASCREEN gliadin ELISA kit	
Evaluation standards	–: <10 mg kg ^{−1} gliadin +: 10–50 mg kg ^{−1} gliadin ++: >50 mg kg ^{−1} gliadin	–: Negative (<10 mg kg ^{−1} gluten) +: positive (10–200 mg kg ^{−1} gluten) ++: high positive (200–10 ⁵ mg kg ^{−1} gluten) +++: very high positive (>10 ⁵ mg kg ^{−1} gluten)	Mean ± SD (mg kg ^{−1})	CV (%)
<i>Gluten-free products</i>				
Pancake mix	(–,–,–)	(–,–,–)	0.34 ± 0.03	8.96
Custard mix	(–,–,–)	(–,–,–)	0.79 ± 0.04	4.87
Chocolate mousse dessert mix	(–,–,–)	(–,–,–)	2.97 ± 0.41	13.96
Chocolate muffin mix	(–,–,–)	(–,–,–)	3.56 ± 0.22	6.23
Tomato cream soup	(–,–,–)	(–,–,–)	4.85 ± 0.19	3.82
Wild mushroom cream soup	(–,–,–)	(–,–,–)	5.08 ± 0.22	4.41
Baby rice	(–,–,+)	(+,+,+)	8.58 ± 0.48	5.62
Kewpie Japanese millet sank	(–,+,+)	(+,+,+)	9.34 ± 0.20	2.12
<i>Wheat-containing products</i>				
Corn cream soup	(+,+,+)	(+,+,+)	11.37 ± 0.35	3.04
Cream cracker	(+,+,+)	(+,+,+)	27.90 ± 0.17	0.61
Fortune cookies	(+,+,+)	(+,+,+)	32.63 ± 3.21	9.84
Plain crackers	(+,+,+)	(+,+,+)	40.91 ± 1.34	3.28
Saltlets brezel	(+,+,+)	(+,+,+)	40.74 ± 1.52	3.74
Digestive biscuit	(+,+,+)	(+,+,+)	41.91 ± 1.26	3.01
Butter cookies	(++,++,+)	(+,+,+)	71.68 ± 5.08	7.08
Wheat toast	(++,++,+)	(+,+,+)	90.34 ± 4.25	4.70

Table 3

Reliability parameters in the detection of different ranges of gliadin concentrations with the developed barcode-style lateral flow immunoassay.

Gliadin range by ELISA kit	Results of the developed assay				Sensitivity	Specificity	Positive predictive value	Negative predictive value	Efficiency	Kappa coefficient
	Positive test results		Negative test results							
	True positive	False positive	True negative	False negative						
<10 mg kg ^{−1}	21	0	24	3	0.88	1.00	1.00	0.89	0.94	0.88
10–50 mg kg ^{−1}	18	3	27	0	1.00	0.90	0.86	1.00	0.94	0.87
>50 mg kg ^{−1}	6	0	42	0	1.00	1.00	1.00	1.00	1.00	1.00

to be US\$3. In this work, the cost per test was estimated to be \$0.49 USD, and the most expensive component was the commercial anti-chicken antibody (which was coated on the control line), as presented in Table S1. The large cost reduction in this study was achieved mainly by the preparation of the anti-gliadin antibody in the laboratory, rather its being bought as a commercial product. A greater reduction in the cost of the manufacturing of strips can be achieved by mass-production in a factory, as opposed to manual production as a batch (Wong & Tse, 2008). Thus, the cheapness of this developed strip assay represents a large step toward its commercialization, and it may indeed become a practical, reliable and cost-effective method for identifying wheat allergens.

4. Conclusions

A rapid barcode-style LFA was developed for the preliminary screening of gliadin in foods. In previous studies, most methods that are based on ELISA have been utilized for quantification and confirmation. The barcode-style LFA that is developed herein has not only the advantages of the lateral flow immunoassay over the ELISA, including its rapidity, user-friendliness, and low-cost, but also provides a semi-quantification test result for preliminary screening purposes. Under optimal conditions, this barcode-style LFA can detect a minimum concentration of 10 mg kg^{−1} gliadin, and it has cut-off values of 10 and 50 mg kg^{−1} gliadin, which can be used to classify “gluten-free” foods (<10 mg kg^{−1} gliadin) and “very low gluten” foods (10–50 mg kg^{−1} gliadin), consistent with the Codex recommendation. In addition, the concept on which this

assay is based can be applied in the semi-quantification of any protein.

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Appendix A. Supplementary data

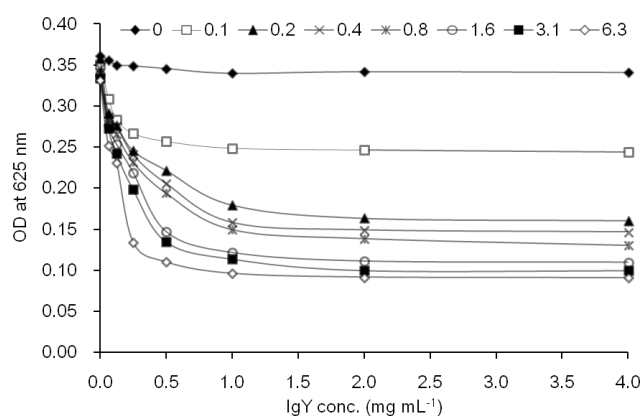
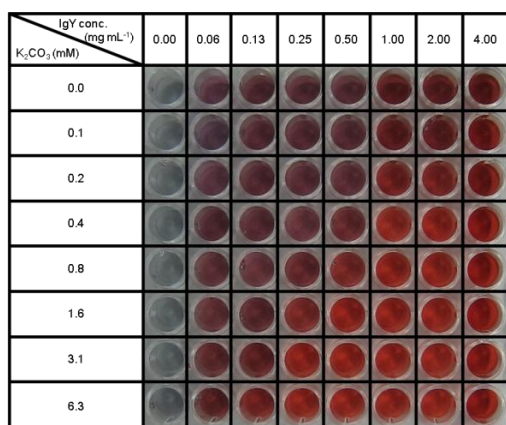
Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2015.06.112>.

References

- Akobeng, A. K. (2007). Understanding diagnostic tests 1: sensitivity, specificity and predictive values. *Acta Paediatrica*, 96, 338–341.
- Chu, P. T., & Wen, H. W. (2013). Sensitive detection and quantification of gliadin contamination in gluten-free food with immunomagnetic beads based liposomal fluorescence immunoassay. *Analytica Chimica Acta*, 787, 246–253.
- Chu, P. T., Lin, C. S., Chen, W. J., Chen, C. F., & Wen, H. W. (2012). Detection of gliadin in foods using a quartz crystal microbalance biosensor that incorporates gold nanoparticles. *Journal of Agriculture and Food Chemistry*, 60, 6483–6492.
- Cohen, J. (1968). Weighted kappa: nominal scale agreement with provision for scaled disagreement or partial credit. *Psychological Bulletin*, 70, 213–220.
- Dzantiev, B. B., Byzova, N. A., Urusov, A. E., & Zherdev, A. V. (2014). Immunochromatographic methods in food analysis. *TrAC-Trends in Analytical Chemistry*, 55, 81–93.

- Fang, C., Chen, Z., Li, L., & Xia, J. (2011). Barcode lateral flow immunochromatographic strip for prostate acid phosphatase determination. *Journal of Pharmaceutical and Biomedical Analysis*, 56, 1035–1040.
- Fasano, A., Berti, I., Gerarduzzi, T., Not, T., Colletti, R. B., Drago, S., et al. (2003). Prevalence of celiac disease in at-risk and not-at-risk groups in the United States: a large multicenter study. *Archives of Internal Medicine*, 163, 268–292.
- Ferrari, G. G., & Eng, P. A. (2011). IgE-mediated food allergies in Swiss infants and children. *Swiss Medical Weekly*, 141, w13269.
- Fung, K. K., Chan, C. P., & Renneberg, R. (2009). Development of enzyme-based bar code-style lateral-flow assay for hydrogen peroxide determination. *Analytica Chimica Acta*, 634, 89–95.
- Kolosova, A. Y., De Saeger, S., Sibanda, L., Verheijen, R., & Van Peteghem, C. (2007). Development of a colloidal gold-based lateral-flow immunoassay for the rapid simultaneous detection of zearalenone and deoxynivalenol. *Analytical and Bioanalytical Chemistry*, 389, 2103–2107.
- Kotaniemi-Syrjänen, A., Palosuo, K., Jartti, T., Kuitunen, M., Pelkonen, A. S., & Mäkelä, M. J. (2010). The prognosis of wheat hypersensitivity in children. *Pediatric Allergy and Immunology*, 21, e421–e428.
- Landis, J. R., & Koch, G. G. (1977). The measurement of observer agreement for categorical data. *Biometrics*, 33, 159–174.
- Leung, W., Chan, C. P., Rainer, T. H., Ip, M., Cauterley, G. W., & Renneberg, R. (2008). Infect check CRP barcode-style lateral flow assay for semi-quantitative detection of C-reactive protein in distinguishing between bacterial and viral infections. *Journal of Immunological Methods*, 336, 30–36.
- Liebers, V., Sander, I., Van Kampen, V., Raulf-Heimsoth, M., Rozynek, P., & Baur, X. (1996). Overview on denominated allergens. *Clinical and Experimental Allergy*, 26, 494–516.
- Liu, G., Mao, X., Phillips, J. A., Xu, H., Tan, W., & Zeng, L. (2009). Aptamer-nanoparticle strip biosensor for sensitive detection of cancer cells. *Analytical Chemistry*, 81, 10013–10018.
- Lou, S. C., Patel, C., Ching, S., & Gordon, J. (1993). One-step competitive immunochromatographic assay for semiquantitative determination of lipoprotein (a) in plasma. *Clinical Chemistry*, 39, 619–624.
- Low, S. C., Shaimi, R., Thandaithabany, Y., Lim, J. K., Ahmad, A. L., & Ismail, A. (2013). Electrophoretic interactions between nitrocellulose membranes and proteins: biointerface analysis and protein adhesion properties. *Colloids and Surface B: Biointerfaces*, 110, 248–253.
- Mao, X., Ma, Y., Zhang, A., Zhang, L., Zeng, L., & Liu, G. (2009). Disposable nucleic acid biosensors based on gold nanoparticle probes and lateral flow strip. *Analytical Chemistry*, 81, 1660–1668.
- Morita, E., Chinuki, Y., Takahashi, H., Nabika, T., Yamasaki, M., & Shiwaku, K. (2012). Prevalence of wheat allergy in Japanese adults. *Allergology International*, 61, 101–105.
- Nassef, H. M., Bermudo Redondo, M. C., Ciclitira, P. J., Ellis, H. J., Fragoso, A., & O'Sullivan, C. K. (2008). Electrochemical immunosensor for detection of celiac disease toxic gliadin in foodstuff. *Analytical Chemistry*, 80, 9265–9271.
- Paek, S. H., Lee, S. H., Cho, J. H., & Kim, Y. S. (2000). Development of rapid one-step immunochromatographic assay. *Methods*, 22, 53–60.
- Palosuo, K., Varjonen, E., Kekki, O. M., Klemola, T., Kalkkinen, N., Alenius, H., et al. (2001). Wheat ω -5 gliadin is a major allergen in children with immediate allergy to ingested wheat. *Journal of Allergy and Clinical Immunology*, 108, 634–638.
- Park, H. J., Kim, J. H., Kim, J. E., Jin, H. J., Cho, G. S., Ye, Y. M., et al. (2011). Diagnostic value of the serum-specific IgE ratio of ω -5 gliadin to wheat in adult patients with wheat-induced anaphylaxis. *International Archives of Allergy and Immunology*, 157, 147–150.
- Pinto, A., Polo, P. N., Henry, O., Redondo, M. C. B., Svobodova, M., & O'Sullivan, C. K. (2014). Label-free detection of gliadin food allergen mediated by real-time apta-PCR. *Analytical and Bioanalytical Chemistry*, 406, 515–524.
- Roth, J. (1982). The preparation of protein A-gold complexes with 3 nm and 15 nm gold particles and their use in labelling multiple antigens on ultra-thin sections. *Histochemical Journal*, 14, 791–801.
- Sampson, H. A. (1999). Food allergy. Part 1: Immunopathogenesis and clinical disorders. *Journal of Allergy and Clinical Immunology*, 103, 717–728.
- Sánchez, D., Tucková, L., Burkhard, M., Plicka, J., Mothes, T., Hoffmanová, I., et al. (2007). Specificity analysis of anti-gliadin mouse monoclonal antibodies used for detection of gliadin in food for gluten-free diet. *Journal of Agriculture and Food Chemistry*, 55, 2627–2632.
- Saraiva, S. M., & de Oliveira, J. F. (2002). Control of particle size in the preparation of colloidal gold. *Journal of Dispersion Science and Technology*, 23, 837–844.
- Sealey-Voyksner, J. A., Khosla, C., Voyksner, R. D., & Jorgenson, J. W. (2010). Novel aspects of quantitation of immunogenic wheat gluten peptides by liquid chromatography-mass spectrometry/mass spectrometry. *Journal of Chromatography A*, 1217, 4167–4183.
- Sunwoo, H. H., Gujral, N., Lutz, S., & Suresh, M. (2012). Double antibody sandwich enzyme linked immunoassay and rapid immunoswab assay for detection of gliadin in food. *Food and Agricultural Immunology*, 23, 169–181.
- Thompson, T., & Méndez, E. (2008). Commercial assays to assess gluten content of gluten-free foods: why they are not created equal. *Journal of the American Dietetic Association*, 108, 1682–1687.
- Valdés, I., García, E., Llorente, M., & Méndez, E. (2003). Innovative approach to low-level gluten determination in foods using a novel sandwich enzyme-linked immunosorbent assay protocol. *European Journal of Gastroenterology and Hepatology*, 15, 465–474.
- Van den Broeck, H. C., America, A. H., Smulders, M. J., Bosch, D., Hamer, R. J., Gilissen, L. J., et al. (2009). A modified extraction protocol enables detection and quantification of celiac disease-related gluten proteins from wheat. *Journal of Chromatography B*, 877, 975–982.
- Venosa, A. D., King, D. W., & Sorial, G. A. (2002). The baffled flask test for dispersant effectiveness: a round robin evaluation of reproducibility and repeatability. *Spill Science and Technology Bulletin*, 7, 299–308.
- Wong, R. C., & Tse, H. Y. (2008). *Lateral flow immunoassay*. New York: Humana Press.
- Yavuz, S. T., Sahiner, U. M., Buyuktiryaki, B., Soyer, O. U., Tuncer, A., Sekerel, B. E., et al. (2010). Phenotypes of IgE-mediated food allergy in Turkish children. *Allergy and Asthma Proceedings*, 32, 47–55.
- Yonekita, T., Ohtsuki, R., Hojo, E., Morishita, N., Matsumoto, T., Aizawa, T., et al. (2013). Development of a novel multiplex lateral flow assay using an antimicrobial peptide for the detection of Shiga toxin-producing *Escherichia coli*. *Journal of Microbiol Methods*, 93, 251–256.
- Zhang, S., Leem, G., Srisombat, L. O., & Lee, T. R. (2008). Rationally designed ligands that inhibit the aggregation of large gold nanoparticles in solution. *Journal of the American Chemical Society*, 130, 113–120.
- Zhou, G., Mao, X., & Juncker, D. (2012). Immunochromatographic assay on thread. *Analytical Chemistry*, 84, 7736–7743.
- Zhou, J., Ralston, J., Sedev, R., & Beattie, D. A. (2009). Functionalized gold nanoparticles: synthesis, structure and colloid stability. *Journal of Colloid and Interface Science*, 331, 251–262.

(A)



(B)

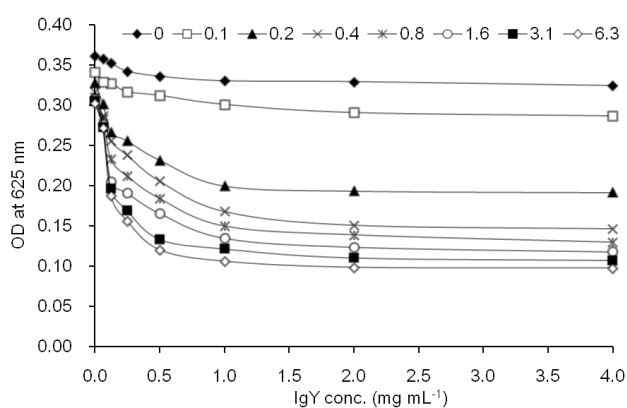
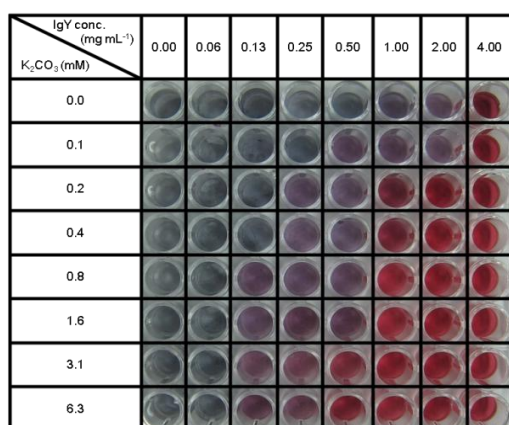


Fig. S1. Optimization of the production of immuno-AuNPs with diameters as (A) 20 nm and (B) 45 nm. The pH of the AuNP solution was initially adjusted by adding various concentrations of potassium carbonate solution, and then different concentrations of anti-gliadin IgY antibodies were mixed with these treated AuNPs to form immuno-AuNPs. To assess the stability of immuno-AuNPs, they were incubated with 10% NaCl for 1 hour and the optical density of the tested samples were measured using an ELISA reader at a wavelength of 625 nm.

Table S1. Estimated material cost of the developed barcode-style lateral flow immunoassay.

Materials	Cost (USD)/ volume	Volume (μ L)/ test	Cost/ test	Total cost/ test
NC membrane	167/5 pic	-	\$ 0.07	
Absorbent pad	8/100 pic	-	\$ 0.0003	
AuNP	6/mL	30	\$ 0.18	\$ 0.49
Anti-chicken antibody (1 mg mL^{-1})	120/mL	1.5	\$ 0.2	
Anti-gliadin antibody (30 mg mL^{-1})	20/mL	2	\$ 0.04	