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Development and Validation of a Lateral Flow Immunoassay Test Kit for Dual Detection of Casein and β-Lactoglobulin Residues

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ABSTRACT

Allergies to cow's milk are very common and can present as life-threatening anaphylaxis. Consequently, food labeling legislation mandates that foods containing milk residues, including casein and/or β-lactoglobulin, provide an indication of such on the product label. Because contamination with either component independent of the other can occur during food manufacturing, effective allergen management measures for containment of milk residues necessitates the use of dual screening methods. To assist the food industry in improving food safety practices, we have developed a rapid lateral flow immunoassay test kit that reliably reports both residues down to 0.01 μg per swab and 0.1 ppm of protein for foods. The assay utilizes both sandwich and competitive format test lines and is specific for bovine milk residues. Selectivity testing using a panel of matrices with potentially interfering substances, including commonly used sanitizing agents, indicated reduction in the limit of detection by one-to fourfold. With food, residues were easily detected in all cow's milk–based foods tested, but goat and sheep milk residues were not detected. Specificity analysis revealed no cross-reactivity with common commodities, with the exception of kidney beans when present at high concentrations (>1%). The development of a highly sensitive and rapid test method capable of detecting trace amounts of casein and/or β-lactoglobulin should aid food manufacturers and regulatory agencies in monitoring for milk allergens in environmental and food samples.

Key words: Allergens; Casein; β-Lactoglobulin; Lateral flow device; Milk; Polyclonal antibodies

Immunotoxicity from exposure to cow's milk is one of the most common causes of food allergies, affecting roughly 3% of the population, and involves both immediate type (immunoglobulin [Ig] E-dependent) and delayed type (IgE-independent) sensitivity reactions. Accordingly, hypersensitivity to milk proteins can present as a complex of symptoms including dermatitis, urticaria, gastrointestinal indications, airway reactivity, and anaphylactic shock *(1–3)*. Although milk contains more than 40 distinct proteins, those of primary clinical significance are casein and β-lactoglobulin (BLG), the major component of milk whey *(7)*. Bovine milk protein has a predicted composition ratio of 80% casein and 10% BLG *(8)*. However, casein and BLG frequently are independently added to foods, supplements, and personal hygiene products as crudely purified casein or whey protein. For example, casein derivatives are often added to wine as a fining agent to optimize organoleptic properties *(9),* and baked goods and protein-fortified foods such as nutritional supplements and infant formulas frequently contain milk whey protein *(4, 5)*. In both instances, crudely purified casein, whey isolate, or a combination of both is added during food manufacturing, thereby enabling the broadcasting of residue and cross-contact with other foods. Consequently, food products labeled as dairy free can become variably contaminated with whey and/or casein residues. Because cross-contact with milk proteins during food processing can result in clinically significant contamination *(6),* a method is needed for simultaneously monitoring both casein and BLG residues during food manufacturing.

Currently, screening strategies for casein and BLG have relied on kits that detect these two allergens independently or simultaneously. The simultaneous, more economical approach is limited by the fact that casein is overreported compared with BLG, thereby causing ambiguity as to the actual scope of "milk" contamination. To address this problem, we have developed a rapid immunochromatographic test in the form of a lateral flow device (LFD) that can be used to screen foods and environmental samples with a limit of detection (LOD) of 0.1 ppm and 0.01 μg per swab, respectively, for both casein and BLG. This dual detection kit would improve allergen control procedures and hazard analysis critical control point systems for food manufacturers and assist regulatory agencies in monitoring for milk allergens in environmental and food testing samples.

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MATERIALS AND METHODS

Reagents and assay buffers. Sodium caseinate and BLG were purchased from Sigma-Aldrich (St. Louis, MO). Nonfat skim milk (NFSM; Kroger, Cincinnati, OH) powder and whey protein isolate (unflavored Isopure, Nature's Best, Brea, CA) were purchased from local stores. Purity of reference materials was >95% based on denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (data not shown). Protein concentration was determined using a bicinchoninic acid protein assay (Thermo Scientific, Wilmington, DE), with bovine serum albumin (BSA) as a reference standard to generate a standard curve. Affinity purified polyclonal antibodies against casein and BLG and sample extraction buffer were obtained from Pi Bioscientific (Seattle, WA). An anti-casein polyclonal antibody (pAb) was generated in a goat, and the anti-BLG pAb was raised in a rabbit using approved protocols. The pAbs were first purified on protein G columns and then subsequently purified on casein- or BLG-conjugated glyoxyl-agarose bead (Agarose Bead Technologies, Tampa, FL) columns using a fast protein liquid chromatography unit (ÄKTAFPLC Prime, GE Healthcare Life Sciences, Pittsburgh, PA). To make protein-agarose columns for affinity purification, agarose beads were glyoxylated, periodate oxidized, and then conjugated to primary amines on target molecules. The ensuing matrix was rinsed and packed into a chromatography column. The commercial sanitizer agents tested were BioSide HS (15%; Enviro Tech Chemical Services, Modesto, CA), Power 99 Plus (Morgan Gallacher, Santa Fe Springs, CA), and Blend Foam Cleaner (Univar, Redmond, WA). BioSide contains acetic acid, peroxyacetic acid, and hydrogen peroxide and is typically used at a working concentration of 200 ppm. Power 99 Plus is a liquid alkaline degreaser that saponifies and emulsifies oils and fatty soils and is supplied as 16 mg/ml or 16,000 ppm. The Univar foam contains strong bases, oxidizing agents, and surfactants and is supplied as 37.12 mg/ml or 37,120 ppm.

Preparation of gold conjugates. Citrate-capped 40-nm gold nanoparticles were obtained from Pi Bioscientific. Anti-casein or anti-BLG IgG was diluted in borate buffer to a final concentration of 0.1 mg/ml, and then 7.5 ml was added drop-wise to 250 ml of gold nanoparticles ($A_{530} = 1$) while stirring for 30 min. To block, 2.5 ml of 10% BSA (in borate buffer) was added, and the colloid was pelleted by centrifugation at 3,000 × *g* for 1.5 h. Spectral analysis was performed on the resuspended soft pellet, and the absorbance was adjusted to a final reading of $A = 20$ (at the absorption maxima) using 1% BSA plus 10% sucrose in 8 mM borate buffer.

Preparation of LFDs. Nitrocellulose membrane (Sartorius, Göttingen, Germany) was lined with a mixture of affinity-purified goat anti-casein and rabbit anti-BLG antibodies for the sandwich format test line (T1), NFSM for the competitive format test line (T2), and chicken anti-goat antibodies for the procedural control line (PC) using an IsoFlow reagent dispenser (Imagene Technology, Hanover, NH). To prepare the conjugate pad, anti-casein and anti-BLG gold conjugates were sprayed on strips of glass fiber conjugate pad material (Ahlstrom, Mt. Holly Springs, PA) using the IsoFlow dispenser. To assemble the test strips, the nitrocellulose membrane, conjugate pad, sample pad (Ahlstrom), and absorbent pad (Advanced Micro Devices, New Delhi, India) were adhered to the adhesive laminate of the backing card (Lohmann, Precision Die Cutting, San Jose, CA) with overlapping surfaces to ensure continuous capillary transfer. The assembled cards were then cut into 5-mm-wide strips using a Matrix 2360 programmable shearer (Kinematic Automation, Sonora, CA), housed in plastic cassettes (Advanced Micro Devices), and stored with desiccant in sealed foil bags at room temperature until used. The LFD was configured such that the sample first encounters the T1 line (a mixture of anti-casein and anti-BLG IgG antibodies), then the T2 line (casein and BLG), and then the PC line (chicken anti-goat IgG antibodies).

In the initial product design, we considered two separate assay configurations, one that combines the BLG and casein signals at the sandwich test line and another that separates these signals as two distinct sandwich test lines. However, we were able to develop the assay so that it essentially reported

$$\text{casein ppm} = \text{BLG ppm} \,(\text{whey ppm}) = \text{total milk ppm}$$

so we opted to advance the former assay format (combined signal) to simplify the interpretation for end users, especially those not using electronic readers, because the response outcome for contamination screening with BLG is no different than that for contamination with casein residues.

Sample preparation. Samples were mixed and homogenized, and 1-g (for solids) or 1-ml (for liquids) aliquots were diluted with 10 and 9 ml, respectively, of extraction buffer. The samples were then extracted at 95°C in a water bath for 1 min, the ensuing extracts were cooled to room temperature and then centrifuged (~2,500 × *g*) for 15 min to promote phase separation, and 100 μl of the aqueous phase was collected and directly applied to the sample port of the LFD.

Assay procedure. Before starting the assay, extraction buffer and LFDs were equilibrated to room temperature. Sample extract (100 μl) was applied to the sample port of the LFD to hydrate the gold conjugate and wick across the nitrocellulose membrane. After 15 min, the results were read using a Qiagen ESE-Quant gold strip reader (Qiagen, Stockach, Germany).

Interpretation of results. Unless otherwise noted, the results reported are the mean (standard deviation [SD]) of three replicates performed by a single analyst. The results of the assay were interpreted as follows. In the absence of analyte (casein or BLG), T1 will not appear but T2 will appear. When the analyte concentration is at or just above the LOD (0.01 ppm of casein or BLG), a clearly visible T1 line will appear with the T2 line. As the concentration of analyte increases (0.1 to 10 ppm), the T1 line will increase in intensity and the T2 line will decrease in intensity. Above 10 ppm of analyte, both T1 and T2 lines will decrease in intensity, with the T2 line disappearing at high analyte concentrations (>100 ppm).

RESULTS AND DISCUSSION

Sensitivity and dynamic range testing. The analytical limit of detection was initially tested using NFSM powder dissolved at serial dilutions in extraction buffer. In each instance, 100 μl of each sample "extract" was applied to the sample port of the LFD and permitted to migrate for 15 min, at which time the test result was read with an electronic strip reader. The threshold for a positive result was set at 60 units, which is the reading when the test line starts becoming clearly visible to the naked eye. At 0 and 0.001 ppm of NFSM, none of the test strips reached the positive threshold; however, 100% of the test strips with 0.01 ppm of NFSM gave a positive result (Table 1). The LFDs were then tested using sodium caseinate dissolved at serial dilutions in extraction buffer. Again, 100 μl of sample was applied, and the results were read at 15 min. At 0 and 0.001 ppm of

TABLE 1. *Analytical sensitivity and dynamic range of the LFD kit for total milk using NFSM as the test analyte*[a]

NFSM (ppm)	Mean (SD) strip reader value: Test line 1 (sandwich)	Mean (SD) strip reader value: Test line 2 (competitive)	Result
Blank	0 (0)	705 (18)	Negative
0.001	55 (4)	714 (28)	Negative
0.01	86 (14)	624 (16)	Positive
0.1	135 (9)	615 (12)	Positive
1	279 (17)	486 (20)	Positive
10	305 (4)	167 (12)	Positive
100	325 (8)	50 (1)	Positive[b]
1,000	169 (19)	0 (0)	Positive[b]

[a] NFSM was dissolved in extraction buffer, 100 μl was applied to the LFD, and results were read at 15 min with a strip reader. Reported values were calculated from triplicate test strips. The threshold for a positive result was set at 60 units.
[b] High concentrations of NFSM resulted in attenuation (strip reader value $<$ 100) of test line 2.

caseinate, none of the test strips produced a positive results, but all of the test strips with 0.01 ppm of caseinate were positive (Table 2). The LFDs also were tested using whey protein isolate powder dissolved in extraction buffer, and the LOD was determined to be 0.01 ppm (Table 3). Accordingly, the overall analytical LOD at the T1 line for all target analytes was 0.01 ppm of protein, which translates to 0.1 ppm in foods because of the incorporation of a 10-fold dilution that is imposed as a consequence of sample preparation. When considering operation across a broad dynamic range (0.001 to 1,000 ppm), the rate of signal decrease at T2 (competitive test format) was faster than that at T1 (sandwich test format) for NFSM and caseinate, whereas the rate of signal decrease on T2 was slightly slower relative to that on T1 for whey protein (compare Tables 1 through 3). However, the T2 line LOD was not consistent for all three targets, with notable reduced sensitivity for

TABLE 2. *Analytical sensitivity and dynamic range of the LFD kit for total milk using sodium caseinate as the test analyte*[a]

Caseinate (ppm)	Mean (SD) strip reader value: Test line 1 (sandwich)	Mean (SD) strip reader value: Test line 2 (competitive)	Result
Blank	0 (0)	651 (14)	Negative
0.001	65 (7)	670 (19)	Negative
0.01	101 (13)	677 (22)	Positive
0.1	136 (9)	596 (38)	Positive
1	175 (12)	452 (12)	Positive
10	184 (5)	314 (8)	Positive
100	227 (24)	110 (14)	Positive
1,000	304 (16)	0 (0)	Positive[b]

[a] Sodium caseinate was dissolved in extraction buffer, 100 μl was applied to the LFD, and results were read at 15 min with a strip reader. Reported values were calculated from triplicate test strips. The threshold for a positive result was set at 60 units.
[b] High concentrations of caseinate resulted in attenuation (strip reader value $<$ 100) of test line 2.

TABLE 3. *Analytical sensitivity and dynamic range of the LFD kit for total milk using whey protein isolate (Isopure) as the test analyte*[a]

Whey protein (ppm)	Mean (SD) strip reader value: Test line 1 (sandwich)	Mean (SD) strip reader value: Test line 2 (competitive)	Result
Blank	0 (0)	674 (21)	Negative
0.001	46 (8)	726 (42)	Negative
0.01	99 (11)	677 (71)	Positive
0.1	143 (22)	696 (34)	Positive
1	204 (4)	688 (35)	Positive
10	166 (16)	667 (73)	Positive
100	88 (14)	449 (37)	Positive
1,000	60 (6)	156 (21)	Positive[b]

[a] Whey protein was dissolved in extraction buffer, 100 μl was applied to the LFD, and results were read at 15 min with a strip reader. Reported values were calculated from triplicate test strips. The threshold for a positive result was set at 60 units.
[b] High concentrations of whey protein resulted in attenuation (strip reader value $<$ 100) of test line 2.

whey protein. This difference in T2 LOD values is a likely consequence of differences in avidity for the two pAbs for the target analyte, where the number of actual pAb binding sites differed from the individual binding affinity for each site on each of the different targets, thus preventing consistency in the LODs of T1 and T2 for both casein and BLG.

Kinetic analysis. The LFDs were assessed at 0 and 0.01 ppm of NFSM, and results were read at 1-min intervals for 15 min with a Qiagen ESE-Quant Gold strip reader. Strip reader values generated at the T1, T2, and PC lines were monitored as a function of time (Fig. 1). With 0.01 ppm of NFSM protein, all three lines were clearly visible and almost plateaued by 15 min, indicating that 15 min is a suitable assay end point. LFDs operated without analyte (0 ppm) did not register any signal at T1 but signal did appear at T2, indicating that the assay is not likely to produce false-positive results and test line artifacts. This feature is important because the commercial LFD (Romer Labs, Union, MO) for total milk that was used for comparison produced strong false-positive signals at the test line when the results were read just after 5 min of operation, causing potential uncertainty in the interpretation of these test results and preventing archiving of test strips and electronic reading of the test result.

Cross-reactivity analysis. To determine the specificity of the assay, full strength extracts were prepared from a panel of selected commodities. Cross-reactivity was detected for kidney bean probably because of the presence of lectins (Table 4). In this instance, the T2 line was unaffected despite the fact that the analyte was tested at full strength. This result suggests that the cross-reactivity was caused by cross-linking of antibodies in both the capture and gold conjugate reagent, presumably because of the lectins in the beans. The cross-reactivity disappeared when the kidney bean extract was diluted 1:100. Analysis of dairy-based foods prepared

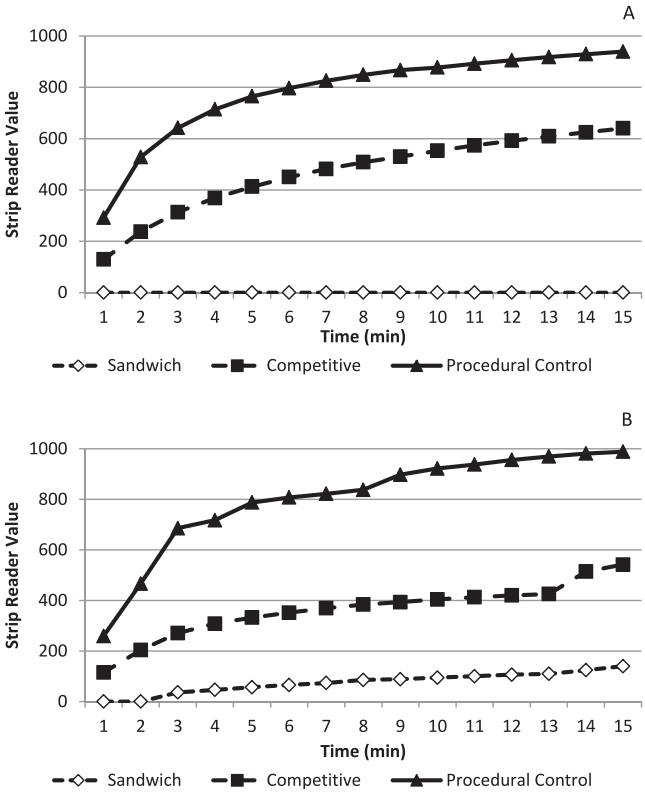

FIGURE 1. *Kinetic analysis. Non-fat skim milk protein (NFSM) at 0 ppm (A) and 0.01 ppm (B) was prepared in extraction buffer and then applied to the LFD and read at 1-min intervals for 15 min with a Qiagen ESE-Quant Gold strip reader. Reported values are means calculated from triplicate tests performed, with each of the three reagent lines read only once.*

from goat's milk and ewe's milk (Table 5) produced negative results, indicating no cross-reactivity with residues from these milks.

Food and spiking recovery analyses. The LFD assay was used to test a panel of foods containing dairy products, including foods that had undergone fermentation or thermal processing and foods with high polyphenol concentrations and exceptionally high fat concentrations. When analyte concentration was high (indicated by disappearance of the T2 line), the samples were diluted further in extraction buffer and retested. With the exception of blueberry muffin, which contained baked whey protein and not casein, foods containing raw bovine milk residues were positive with the LFD assay at up to 100,000-fold dilution, on par with the dilutions used for nonproblematic foods such as skim milk and partially skim milk (Table 5). Full strength extracts of yogurt and cheese prepared with nonbovine milk produced no signals.

Matrix effects. The LFD assay was evaluated for selectivity by spiking a panel of complex foods with increasing concentrations of NFSM protein at increments of the LOD (0.1, 0.2, and 0.4 ppm). The six model foods were selected because they present distinct challenges to allergen recoverability, assay fluidics, and immune-based detection of allergen residues, including high polyphenol concentrations, increased osmolarity and ionic strength, high protein concentrations, high fat concentrations, low pH, and high viscosity. Spiking known amounts of target analyte into complex matrices enabled a more comprehensive assessment of the effectiveness of the buffer extraction method for recovery of the target analyte. Given the homogeneous assay format for LFD and the limitations with respect to matrix effects, this approach allowed determination of how complicated matrices impact the performance of the device regarding fluidics and antigen-antibody immune complex formation. Dark chocolate (70% cocoa), with an inherently high phenol concentration, had an LOD of 0.4 ppm (Table 6). The other test foods produced positive results between 0.1 and 0.2 ppm of NFSM. Thus, the overall assay remained sensitive in foods with potentially interfering substances at concentrations of less than 0.5 ppm, thereby indicating reasonable resistance of the assay to matrix effects.

TABLE 4. *Specificity analysis*[a]

Food (dilution)	Mean (SD) strip reader value		Result
	Test line 1 (sandwich)	Test line 2 (competitive)	
Lima bean	0	566	Negative
Lupin	0	605	Negative
Kidney bean	482 (16)	650 (44)	Positive[b]
Kidney bean (1/10)	145 (11)	639 (66)	Positive[b]
Kidney bean (1/100)	42 (7)	794 (59)	Negative[b]
Adzuki bean	0	562	Negative
Coconut	0	667	Negative
Chick pea	0	599	Negative
Poppy seed	27	588	Negative
Banana	0	565	Negative
Apple	0	604	Negative
Raw chicken	0	590	Negative
Raw beef	0	568	Negative
Sesame seed	0	574	Negative
Peanut	0	561	Negative
Almond	0	530	Negative
Brazil nut	0	612	Negative
Macadamia nut	20	694	Negative
Pine nut	0	564	Negative
Walnut	0	506	Negative
Hazelnut	0	641	Negative
Cashew nut	36 (5)	661 (27)	Negative[b]
Pistachio nut	41 (8)	661 (70)	Negative[b]
Soybean	0	659	Negative
Celery seed	0	444	Negative
Mustard	0	518	Negative
Milk	62	0	Positive[c]
Egg	0	236	Negative

[a] Full strength extracts were prepared from a panel of foods using extraction buffer and then applied directly to the LFD. Results were read with a strip reader after 15 min. Negative results were obtained from a single measurement; positive results (mean, SD) were calculated from triplicate test strips. The threshold for a positive result was 60 units.

[b] Nonmilk commodities that registered $\geq$35 units at test line 1 were tested in triplicate.

[c] High concentrations of analyte resulted in attenuation (strip reader value $<$ 100) of test line 2.

TABLE 5. *Food analysis*[a]

Food (dilution)	Mean (SD) strip reader value: Test line 1 (sandwich)	Mean (SD) strip reader value: Test line 2 (competitive)	Result
Blank	0	648 (47)	Negative
Whipping cream (1/10)	316 (32)	39 (13)	Positive
Whipping cream (1/100,000)	77 (7)	677 (7)	Positive
Chocolate milk (1/10)	292 (8)	0	Positive
Chocolate milk (1/100,000)	79 (18)	619 (18)	Positive
Evaporated milk (1/10)	125 (21)	6 (10)	Positive
Evaporated milk (1/100,000)	82 (15)	665 (22)	Positive
Mango pineapple yogurt (1/10)	281 (28)	26 (18)	Positive
Mango pineapple yogurt (1/100,000)	77 (3)	638 (24)	Positive
Blueberry muffin with whey (1/10)	202 (75)	360 (26)	Positive
String cheese (1/10)	118 (38)	0	Positive
String cheese (1/100,000)	178 (23)	482 (19)	Positive
Goat cheese (1/10)	22 (21)	418 (28)	Negative
Natural salted butter (1/10)	378 (22)	13 (22)	Positive
Natural salted butter (1/100,000)	89 (12)	613 (7)	Positive
Sheep yogurt (1/10)	7 (12)	431 (37)	Negative
Skim milk (1/10)	107 (6)	0	Positive
Skim milk (1/100,000)	244 (41)	484 (37)	Positive
2% reduced fat milk (1/10)	104 (16)	0	Positive
2% reduced fat milk (1/100,000)	260 (20)	462 (38)	Positive
Half & half (1/10)	124 (10)	0	Positive
Half & half (1/100,000)	234 (15)	544 (49)	Positive
Heavy whipping cream (1/10)	240 (16)	19 (19)	Positive
Heavy whipping cream (1/100,000)	171 (17)	555 (9)	Positive

[a] Dairy-containing commodities were extracted using the extraction buffer and tested at 1/10 and 1/100,000 dilutions. Results were calculated from triplicate test strips.

Because allergen contamination often occurs through cross-contact during food manufacturing, effective allergen control measures rely on the use of rapid screening methods that are resistant to the effects of sanitizing agents. To assess the robustness of the total milk LFD to the effects of sanitizer agents, a degreaser, a foam, and BioSide were individually combined with NFSM protein and tested (Table 7). The total milk LFD kit had an analytical LOD of 0.02 μg/ml (ppm) of milk protein at the highest concentrations of sanitizers tested (200 to 1,000 ppm), indicating that the assay was relatively resistant to the effects of residual sanitizer (compared with 0.01 ppm under matrix-free test conditions).

Method concordance. Two foods (raw pork sausage and pasteurized orange juice) and rinse water (tap water) were spiked with low concentrations (0.2 and 2 ppm) of NFSM protein, caseinate, and whey (Isopure) protein. These matrices were selected because they present a challenge to the assay (high fat concentrations, polyphenol concentration, and pH) or because of their functional utility (rinse water for environmental testing). The spiked samples were then tested using two commercial LFD assays for total milk (Reveal 3-D for Total Milk Allergen kit, Neogen, Lansing, MI; and AgraStrip Total Milk kit, Romer Labs) for comparison purposes. Our LFD was tested in triplicate for each target in each food at each level, whereas the commercial tests were assessed only once. The three kits were operated according to the manufacturer's instructions. For the three selected matrices tested using all three target analytes, our LFD assay was consistently more sensitive than the Neogen kit and equal to or better than the Romer kit (Table 8). The use of the calibrated strip reader allowed the test to be assessed objectively, which is key for auditors and industry accreditation.

The incorporation of the competitive test with the sandwich test in our LFD assay enables the end user to identify hydrolyzed residues, which are poorly detected with the sandwich test format, and the PC line allows the operator to determine whether the test was valid, e.g., whether it ran correctly or whether sample migration and/or antibody-antigen interaction occurred as expected. Although the LOD for the T1 (sandwich) line was relatively consistent for the different matrices, the LOD for the T2 (competitive) line was variable among these matrices. The competitive test, which is interpreted as loss of signal with increasing presence of target analyte, is especially prone to false-positive results because the matrix can retard the flow of proteins across the membrane, affecting the interpretation of the results. The simplest way to overcome the inherent problems associated with various matrices and the competitive assay is to use sample extract dilutions. Although this approach reduces the overall sensitivity of the assay by the dilution factor, it reduces the effects of the matrix on the fluidics. For operations that require more careful interpretation of the T2 line, sample extract dilution is recommended.

Although casein and BLG occur in bovine milk at relatively consistent concentrations, their co-occurrence in food items as an ingredient or contaminant can be uncoupled because of the common practice of adding

TABLE 6. *Selectivity (spiking) analysis*[a]

Commodity	Spiking level (NFSM)	Mean (SD) strip reader value: Test line 1 (sandwich)	Mean (SD) strip reader value: Test line 2 (competitive)	Result
70% Chocolate	Blank	0	104	Negative
	LOD	0	206	Negative
	2× LOD	0	168	Negative
	4× LOD	69 (8)	518 (8)	Positive[b]
Orange juice	Blank	0	550	Negative
	LOD	0	544	Negative
	2× LOD	56 (2)	622 (21)	Negative[b]
	4× LOD	115 (12)	578 (28)	Positive[b]
Soy milk	Blank	0	553	Negative
	LOD	68 (3)	553 (32)	Positive[b]
Pasta	Blank	0	589	Negative
	LOD	66 (4)	641 (12)	Positive[b]
Soup base	Blank	0	404	Negative
	LOD	83 (14)	678 (44)	Positive[b]
Vinaigrette salad dressing	Blank	0	404	Negative
	LOD	0	530	Negative
	2× LOD	56 (9)	640 (6)	Negative[b]
	4× LOD	69 (4)	648 (23)	Positive[b]

[a] Performance of the LFD kit for total milk was evaluated by spiking six complex foods with NFSM protein at increments of the LOD (0.1 ppm in food), testing the sample extracts, and then reading the results using a strip reader. Results were calculated as the mean (SD) from triplicate test strips. The threshold for a positive result was 60 units. The analytical LOD was 0.01 ppm.
[b] Matrix–spike level combinations that registered ≥35 units at test line 1 were tested in triplicate.

TABLE 7. *Effects of sanitizers on LFD sensitivity*[a]

Sanitizer	Residue level (ppm)	Spiking level (NFSM)	Mean (SD) strip reader value: Test line 1 (sandwich)	Mean (SD) strip reader value: Test line 2 (competitive)	Result
BioSide	200	LOD	12 (20)	194 (21)	Negative
		2× LOD	39 (2)	195 (28)	Weak positive
	50	LOD	45 (9)	192 (4)	Weak positive
		2× LOD	65 (19)	193 (16)	Positive
	20	LOD	52 (9)	205 (13)	Positive
		2× LOD	51 (7)	197 (29)	Positive
Degreaser	1,000	LOD	31 (4)	203 (6)	Negative
		2× LOD	49 (6)	184 (9)	Weak positive
	250	LOD	33 (32)	198 (6)	Negative
		2× LOD	51 (10)	184 (6)	Positive
	50	LOD	13 (23)	199 (16)	Negative
		2× LOD	42 (3)	194 (5)	Weak positive
Foam	1,000	LOD	33 (29)	197 (16)	Negative
		2× LOD	56 (13)	204 (25)	Positive
	250	LOD	13 (23)	197 (22)	Negative
		2× LOD	52 (1)	186 (10)	Positive
	50	LOD	50 (3)	203 (11)	Positive
		2× LOD	46 (5)	204 (48)	Positive
Buffer only		Blank	0 (0)	235 (14)	Negative
		LOD	57 (3)	187 (14)	Positive
		2× LOD	69 (7)	196 (9)	Positive

[a] Sanitizers were diluted to indicated levels, and 100 μl was combined with 900 μl for 1× LOD and 2× LOD values of NFSM protein and tested on the LFD. Results were calculated as the mean (SD) from triplicate test strips.

TABLE 8. *Comparison of commercial and new LFD kits for identification of spiked analytes*[a]

Commodity	Spike	Concn (ppm)	Results		
			Neogen kit (LOD = 5 ppm)	Romer kit (LOD = 1 ppm)	New LFD kit (LOD = 0.1 ppm)[b]
Orange juice	Blank	0	NT	NT	Negative
	Whey	0.2	Negative	Negative	Positive (61, 66)
	Whey	2	Negative	Positive	Positive (161, 5)
	Casein	0.2	Negative	Negative	Negative (0, 0)
	Casein	2	Negative	Positive	Positive (66, 8)
	Milk	0.2	Negative	Negative	Positive (103, 17)
	Milk	2	Negative	Negative	Positive (124, 12)
Tap water	Blank	0	NT	NT	Negative (0, 0)
	Whey	0.2	Negative	Positive	Positive (64, 7)
	Whey	2	Negative	Positive	Positive (172, 8)
	Casein	0.2	Negative	Negative	Negative (0, 0)
	Casein	2	Positive	Positive	Positive (66, 4)
	Milk	0.2	Negative	Negative	Negative (0, 0)
	Milk	2	Positive	Positive	Positive (69, 7)
Raw pork sausage	Blank	0	NT	NT	Negative (0, 0)
	Whey	0.2	Negative	Positive	Positive (77, 6)
	Whey	2	Negative	Positive	Positive (155, 5)
	Casein	0.2	Negative	Positive	Positive (85, 0)
	Casein	2	Negative	Positive	Positive (182, 14)
	Milk	0.2	Negative	Negative	Negative (0, 0)
	Milk	2	Positive	Negative	Positive (72, 6)
Buffer only	Whey	0.1	NT	NT	Positive (138, 20)
	Casein	0.1	NT	NT	Positive (135, 8)
	Milk	0.1	NT	NT	Positive (137, 9)

[a] Performance of our new LFD for total milk protein was compared with that of two commercial LFD kits using low spiking levels of whey protein, caseinate, and NFSM protein. Qualitative results are reported for the commercial LFD tests based on single replicates, and qualitative and quantitative results are reported for our LFD test based on triplicate test strips. The threshold for a positive result with the Neogen Reveal 3-D Total Milk Allergen kit and candidate assays was 35 units, corresponding to a RANN score of 2 for the Romer Labs AgraStrip assay. For our LFD and the Neogen kit, the thresholds for a positive result with the strip reader were 60 units at the sandwich test line (T1) and <100 units at the competitive test line (T2). The threshold for a positive result using the AgraStrip kit was set at a RANN score of ≥ 2. NT, not tested.

[b] LFD tests were run in triplicate. Quantitative values (mean, SD) are for the T1 line for each spike-matrix combination.

crudely purified casein or whey proteins to foods to enhance the protein content or improve the organoleptic properties. Consequently, screening environmental and food samples for dairy residues as part of an allergen control program requires analytical tools that can report the presence of both casein and BLG residues with the same level of sensitivity. The use of screening tests that fail to meet this requirement can result in inadvertent underreporting of these allergens. Thus, our LFD assay was designed to detect casein and BLG residues down to an analytical sensitivity of 0.01 μg/ml and 0.1 ppm in foods, with limited matrix interference.

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