FOOD COMPOSITION AND ADDITIVES

Validation of the RIDASCREEN®FAST Milk Kit

AOAC *Performance Tested Method*SM 101501

Abstract

The RIDASCREEN®FAST Milk test is a sandwich ELISA for the rapid quantification of milk proteins in various foods. The specific antibodies target casein and β-lactoglobulin. Samples are extracted and can then be analyzed in less than 40 min. The calibration curve covers a range from 2.5 to 67.5 mg/kg milk protein. The assay was validated with cookies, infant formula, chocolate dessert, ice cream, and sausages. All negative samples were found well below the LOQ of 2.5 mg/kg. Recoveries of the spiked samples were mostly in the range of 80–120%. The LOD of the ELISA was found below 1 mg/kg. The analysis of 39 different substances of interest revealed that no cross-reactivity above the LOQ occurred. Ruggedness testing proved that variations in incubation temperature, reagent volume, incubation time, extraction temperature, and extraction time had no significant influence. The stability at 4–8°C of three independent lots was investigated and found to exceed 18 months. Very good lot-to-lot consistency and no significant loss of the analytical capacity over the shelf life were observed. Incurred cookies and chocolate dessert samples were prepared and analyzed by an independent laboratory; mean recoveries of 94.4 and 102.2% and mean SDs of 10.9 and 6.3%, respectively, were found. For the 0 mg/kg level for both materials, all samples tested returned values of <2.5 mg/kg. Therefore, the analytical performance claims of the manufacturer were confirmed.

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Scope of Method

The RIDASCREEN®FAST Milk test is a sandwich ELISA that detects the two major allergenic proteins in milk: casein and β-lactoglobulin. They are detected independently by specific antibodies. The assay results are quantitative when the sample contains milk or milk powder. If the milk protein composition of the sample is not known and the milk protein ratio is different than the natural ratio in milk (e.g., a sample primarily consisting of whey), the results may be underestimated. The method is suitable for determination of milk proteins in cookies, infant formula, chocolate dessert, ice cream, and sausages.

General Information

The milk proteins casein and β-lactoglobulin are known as major causes of severe allergenic reactions. Milk-allergic consumers are therefore dependent on milk-free products. However, milk is present as an ingredient or as cross contamination in a wide variety of foods. When used as an ingredient, milk must be labeled on prepacked products in many countries, including the United States and those in the European Union. Compliance with the legislation is controlled by food manufacturers and independent laboratories, which are in need of reliable test systems to detect small traces of milk in foods. The RIDASCREEN®FAST Milk test is a fast, simple, and sensitive screening method that might help to reduce the danger of allergic cases from mislabeled or contaminated food. The assay result is expressed as milligrams per kilogram milk protein.

Principle

The basis of the test is an antigen-antibody reaction. Specific antibodies against casein and β-lactoglobulin are used for the detection. A microtiter plate is coated with both antibodies as

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capture antibodies. Skim milk powder calibrators [traceable to National Institute of Standards and Technology (NIST) whole milk powder SRM1549a] and sample extracts are incubated for 10 min. After washing, a mixture of the anti-casein and anti-β-lactoglobulin antibody enzyme conjugates is added for a further 10 min. This conjugate binds to the milk protein–antibody complex on the plate (sandwich enzyme immunoassay). Any unbound enzyme conjugate is then removed by a washing step. Substrate/chromogen is added to the wells and incubated for 10 min. Bound enzyme converts the substrate/chromogen to a blue product. The addition of a stop reagent inhibits the enzymatic process and causes a shift of the colored product to yellow. Measurement is performed photometrically within 10 min after stopping, at 450 nm against air. The resulting absorbance values are proportional to the concentration of milk protein in the sample.

Materials and Methods

Test Kit Information

(a) *Kit name*.—R-Biopharm RIDASCREEN®FAST Milk (Cat. No. R4652).

(b) *Ordering information*—(*1*) *United States*.—R-Biopharm, Inc., 870 Vossbrink Dr, Washington, MO 63090; Tel: (877)-789-3033; Fax (866)-922-5856; e-mail: sales@r-biopharm.com.

(*2*) *Outside United States*.—R-Biopharm AG, An der neuen Bergstrasse 17, D-64297 Darmstadt, Germany; Tel: 0049-(0)6151-8102-0; Fax: 0049-(0)6151-8102-20; e-mail: sales@r-biopharm.de.

(c) Further information is available at the Web site address: www.r-biopharm.com.

(d) *Test kit components*.—(*1*) *Microtiter plate*.—Coated with anti-casein and anti-β-lactoglobulin antibodies (48 wells).

(*2*) *Five standards*.—1.3 mL Each of 0, 2.5, 7.5, 22.5, and 67.5 mg/kg milk protein in aqueous solution in ready-to-use transparent capped bottles.

(*3*) *Conjugate*.—Mix of horseradish peroxidase–labeled anti-casein and anti-β-lactoglobulin antibody conjugate (0.7 mL), as an 11× concentrate in a red-capped bottle.

(*4*) *Conjugate buffer*.—7 mL In a ready-to-use black-capped bottle.

(*5*) *Extractor 2*.—Three 30 mL bottles, as a 2× concentrate in a blue-capped bottle.

(*6*) *Allergen Extraction buffer*.—100 mL, As a 10× concentrate in a green-capped bottle.

(*7*) *Wash buffer*.—100 mL, As a 10× concentrate in a brown-capped bottle.

(*8*) *Substrate/chromogen*.—10 mL In a ready-to-use brown-capped bottle.

(*9*) *Stop solution*.—14 mL In a ready-to-use yellow-capped bottle.

(*10*) *Additive 1*.—2 g In a blue-capped bottle, to be dissolved in Allergen Extraction buffer.

Additional Supplies and Reagents

(a) *Micropipets ranging from 20 to 200 µL and 200 to 1000 µL.*

(b) *Pipets for 4 and 16 mL volume pipetting.*

(c) *Graduated cylinders, 100 mL and 1 L, plastic or glassware.*

(d) *Distilled water.*

(e) *1 M sodium hydroxide (NaOH).*

(f) *1 M hydrochloric acid (HCl).*

(g) *Centrifugal vials.*

(h) *Paper filter.*

Apparatus

(a) *Temperature-controlled water bath at 100°C*.—Gesellschaft für Labortechnik (GFL; Burgwedel, Germany).

(b) *Temperature-controlled water bath at 60°C*.—GFL.

(c) *Centrifuge*.—Minifuge RF (Kendro, Hanau, Germany).

(d) *Microtiter plate reader*.—Tecan Sunrise Remote (Tecan Group, Maennedorf, Switzerland).

Standard Reference Materials

The calibrator material used in the test kit is a skim milk powder from MoniQA (MoniQA Association, Vienna, Austria; www.moniqa.org). This material is not yet commercially available but will be circulated in winter 2015/2016. The certified protein content of this material is 35.0–35.8%. For calculation a mean concentration of 35.25% is assumed.

This calibrator is traceable to NIST whole milk powder SRM1549a (LGC Promochem GmbH, Wesel, Germany; www.lgcpromochem.com). The protein content of this material is 25.64 ± 0.31%.

Spike Solution Preparation

Dissolve the milk powder at a concentration of 1 mg/mL protein in urea–phosphate-buffered saline–Tween buffer [preparation of 500 mL: 180.19 g urea ultra-pure (ICN Biomedicals, Eschwege, Germany), 3.4 g sodium chloride, 0.74 g disodium hydrogen phosphate, 0.22 g potassium dihydrogen phosphate, 0.5 g Tween 20, NaOH/HCl for pH adjustment to 8.4]. Dilute the solution further using diluted Allergen Extraction buffer.

Safety Precautions

(a) *β-Mercaptoethanol*.—Extractor 2, necessary for sample preparation, contains β-mercaptoethanol. Use a chemical hood for sample preparation.

(b) *Sulfuric acid*.—Stop solution contains 1 N sulfuric acid. Avoid skin and eye contact.

General Preparation

(a) Allergen Extraction buffer (100 mL) is provided as a 10-fold concentrate. Before dilution, dissolve any formed crystals by heating (water bath at 37°C) and mix well. Dilute the heated concentrate 1:10 (1 + 9) with distilled water (e.g., 100 mL concentrate + 900 mL distilled water). The diluted Allergen Extraction buffer can be used for approximately 12 weeks at 2–8°C/35–46°F.

(b) For the preparation of the Allergen Extraction buffer containing Additive 1 (A-AEP buffer), weigh 1.35 g Additive 1 in a glass beaker and add 15 mL 1 M NaOH. Stir until Additive 1 is dissolved. To 700 mL diluted Allergen Extraction buffer in

a measuring cylinder add the 15 mL Additive 1 solution by stirring constantly, transferring any residues of the Additive 1 solution into the measuring cylinder by rinsing with diluted Allergen Extraction buffer. Adjust the A-AEP buffer to pH 9.0 with 1 M HCl and dilute to 750 mL with diluted Allergen Extraction buffer. 750 mL A-AEP buffer is sufficient for 45 samples. The buffer can be used for approximately 3 weeks at room temperature (20–25°C/68–77°F) or for 8 weeks at 2–8°C/35–46°F (if crystals precipitate, the buffer must be discarded).

(c) The Extractor 2 (three bottles with 30 mL each) is provided as a twofold concentrate and has to be diluted 1:2 (1 + 1) with distilled water (e.g., 50 mL Extractor 2 + 50 mL distilled water). The complete diluted Extractor 2 solution is sufficient for 55 samples and can be used for approximately 3 months at room temperature (20–25°C/68–77°F).

(d) Wash buffer (100 mL) is provided as a 10-fold concentrate. Before use, the buffer has to be diluted 1:10 (1 + 9) with water (e.g., 100 mL buffer concentrate + 900 mL distilled water). The diluted buffer is stable at 2–8°C/35–46°F for 4 weeks. Before dilution, dissolve completely any formed crystals in a water bath at 37°C/99°F.

(e) Heat one water bath to 100°C/212°F and a second water bath to 60°C/140°F. Place the A-AEP buffer in the 60°C/140°F water bath for preheating.

Sample Preparation

(a) Store samples in a cold and dry room protected from light.

(b) Milk protein residues of previous analyses must be removed completely. Therefore, samples should be prepared in new or very well cleaned vials.

(c) Tools such as mincers must be cleaned thoroughly after use for the next sample to avoid spreading traces of milk protein.

(d) Keep in mind that the sample can be inhomogeneous; therefore, grind a representative part of the sample very well and homogenize before weighing.

(e) Sample extracts not yet diluted with Allergen Extraction buffer can be stored at 2–8°C/35–46°F for 3 days. The extracts can be stored at −20°C/−4°F for several months.

Sample Preparation Procedures

(a) *Homogenize a representative amount of the sample (5–50 g).*—(*1*) *Solid samples*.—Weigh 1 g sample and to it add 4 mL diluted Extractor 2. Mix vigorously, close the vial, and cook for 10 min at 100°C/212°F in a water bath. Let the sample cool down shortly and add 16 mL 60°C/140°F preheated A-AEP buffer to the cooked sample. Continue with Section **b**.

(*2*) *Liquid samples*.—To 1 mL sample add 4 mL prepared Extractor 2. Mix vigorously, close the vial, and cook for 10 min at 100°C/212°F in a water bath. Let the sample cool down shortly and add 15 mL 60°C/140°F preheated A-AEP buffer to the cooked sample. Continue with Section **b**.

(b) *Further preparation for solid and liquid samples*.—Mix vigorously (shaker) and extract for 10 min at 60°C/140°F in a water bath. Cool down the samples to room temperature (e.g., using ice water) and centrifuge for 10 min at 2500 × *g* at room temperature (alternatively, 2 mL extract can be centrifuged at high speed for 10 min in reaction caps by using a microcentrifuge). Filter the supernatant using fluted paper filter. Dilute the sample 1:5 (1 + 4) with finally diluted Allergen Extraction buffer not containing Additive 1 (e.g., 100 µL sample + 400 µL diluted Allergen Extraction buffer; final dilution factor is 100). Use 100 µL of this dilution per well directly in the assay.

Analysis

(a) *General recommendation for good test performance*.—(*1*) Bring all reagents to room temperature (20–25°C/68–77°F) before use.

(*2*) Return all reagents to 2–8°C/35–46°F immediately after use.

(*3*) Do not allow microwells to dry between working steps.

(*4*) Reproducibility in any ELISA is largely dependent on the consistency with which the microwells are washed. Carefully follow the recommended washing sequence as outlined in the ELISA test procedure.

(*5*) Avoid direct sunlight during all incubations; covering the microtiter plates is recommended.

(*6*) Substrate/chromogen reaction should be carried out in the dark.

(*7*) Each calibrator and sample should be analyzed in duplicate.

(*8*) Use both milk protein–free (unspiked) and milk protein–containing (spiked) samples as test controls.

(b) *Preparation of ELISA testing*.—The (antibody enzyme) conjugate (bottle with red cap) is provided as an 11-fold concentrate. Because the diluted enzyme conjugate solution has a limited stability, only the amount that is needed should be diluted shortly before the ELISA testing. Shake the conjugate concentrate carefully before diluting. For reconstitution, the conjugate concentrate is diluted 1:11 (1 + 10) with conjugate dilution buffer (e.g., 200 µL concentrate + 2 mL buffer, sufficient for 2 microtiter strips).

(c) *ELISA testing*—(*1*) Insert a sufficient number of wells into the microwell holder (not more than 3 strips per run for all calibrators and samples to be run). In the case of more than three strips, an uncoated plate (e.g., low-binding from Greiner Bio-One, Frickenhausen, Germany, Cat. No. 655101) should be used as a preplate to avoid a time shift over the microtiter plate. All calibrators and samples are pipetted onto the uncoated plate (at least 100 µL/well) and then quickly transferred to the coated microtiter plate with an 8-channel pipet. Record calibrator and sample positions.

(*2*) Add 100 µL of each calibrator solution or prepared sample to separate wells, and incubate for 10 min at room temperature (20–25°C/68–77°F).

(*3*) Pour the liquid out of the wells and tap the microwell holder upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. Fill all wells with 250 µL wash buffer and pour out the liquid again. Repeat three more times.

(*4*) Add 100 µL diluted enzyme conjugate to each well. Mix gently by rocking the plate manually, and incubate for 10 min at room temperature (20–25°C/68–77°F).

(*5*) Pour the liquid out of the wells and tap the microwell holder upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. Fill all wells with 250 µL wash buffer and pour out the liquid again. Repeat three more times.

(*6*) Add 100 µL reddish substrate/chromogen solution to each well. Mix gently by rocking the plate manually, and incubate for 10 min at room temperature (20–25°C/68–77°F) in the dark.

(*7*) Add 100 µL Stop solution to each well. Mix gently by rocking the plate manually, and measure the absorbance at 450 nm against an air blank. Read within 10 min after the addition of Stop solution.

Interpretation and Test Result Report

(a) *Result calculation*.—Special software RIDA®SOFT Win (Art. No. Z9999) is available and recommended for evaluation of RIDASCREEN ELISAs. The calculation should be done using a cubic spline function. When working in accordance with the described sample preparation, the final dilution factor is 100. In this case, the milk protein concentration can be read directly from the calibration curve; the sample dilution factor of 100 is already taken into account for the calibrator concentrations. For sample dilutions of more than 1:100, the additional dilution factor must be considered for the calculation of the milk protein concentration. A further dilution and new detection of the samples is recommended for absorbance values (at 450 nm) greater than Calibrator 5. The samples should be diluted so that the results can be read within the calibration curve. Extrapolation outside the calibration range is not recommended.

(b) *Result reporting*.—Results are reported in milligrams of milk protein per kilogram or liter of food.

(c) *Milk conversion factor*.—Milk contains approximately 3.2% milk protein. Thus, a sample that contains 1 mg/kg milk protein corresponds to a milk content of approximately 32 mg/kg.

Result Interpretation

The course of the calibration curve is shown in the QA certificate enclosed in the test kit. Absolute absorbance may vary between different runs (e.g., due to different temperatures or analysts). However, the shape of the calibration curve should be similar to the one given in the QA certificate. In comparison with the certificate, higher values of absorbance (at 450 nm) for the calibration curve, especially for the unspiked calibrator, may be a result of insufficient washing, wrong antibody conjugate dilution, or milk protein contamination.

Samples with a negative test result still could contain a milk protein contamination below the LOQ of the assay, or they might contain other milk components such as lipids or sugars (e.g., lactose).

Validation Studies

The validation study was conducted according to the AOAC Research Institute (RI) *Performance Tested Method*SM program and the AOAC "International Guideline for Validation Procedures for Quantitative Food Allergen ELISA Methods: Community Guidance and Best Practices" (1). Method developer studies were conducted by R-Biopharm AG (Darmstadt, Germany). The independent-laboratory study was conducted by Q Laboratories (Cincinnati, OH), where a matrix study was undertaken on two of the claimed food matrixes.

LOD

The LOD was calculated according to the guidelines in ref. 1 for nonlinear methods. Five replicates of unspiked samples, as well as spiked samples at four different levels (2.5, 5, 10, and 30 mg/kg) were tested by three different analysts in three different runs using different equipment (pipets, photometers, etc.). Two matrixes were tested: cookies were chosen as a very common matrix for milk testing, and a chocolate dessert as a potentially difficult matrix. This design resulted in 150 measurements in total. Each calibrator and each sample was tested in duplicate (Tables 1 and 2). Mean values and SDs of all samples with the same spike level from the three different analysts were calculated per matrix. Because the RIDA®SOFT Win software of the kit manufacturer does not reliably calculate results below Calibrator 2, a second-order polynomial fitted to Calibrator 1 to Calibrator 3 was used to calculate samples with concentrations less than Calibrator 2 (2.5 mg/kg). The approximation with the second-order polynomial is especially better for quantification near Calibrator 1 than an extrapolated cubic spline function of the RIDA SOFT Win (2). However, it should be noticed that in any case, only concentrations within the calibration curve (i.e., higher than Calibrator 2, with 2.5 mg/kg milk protein) can be reliably quantified per definition (*see* also *LOQ* section).

The SDs were plotted against the sample concentrations, and linear regression was calculated. The following equation from ref. 1 was used for LOD calculation:

$$\text{LOD} = (\text{xbar}(0) + 3.3 \times \text{intercept})/(1 - 1.65 \times \text{slope})$$

Table 1. LOD calculation for chocolate dessert[a]

Chocolate dessert	Blank matrix	Spiked at 2.5 mg/kg	Spiked at 5 mg/kg	Spiked at 10 mg/kg	Spiked at 30 mg/kg
Analyst 1	0.1	2.8	5.1	11.1	35.4
	0.2	2.7	4.8	11.2	35.8
	0.0	2.6	5.0	10.9	33.9
	0.1	2.6	5.2	11.4	31.1
	0.0	2.6	4.7	11.1	31.0
Analyst 2	0.2	2.8	5.4	13.3	32.8
	0.2	2.7	5.1	13.1	33.6
	0.1	2.8	4.9	13.5	34.3
	0.1	2.7	5.1	13.8	32.9
	0.0	2.8	5.0	14.2	33.0
Analyst 3	0.3	3.2	6.3	12.8	32.1
	0.5	3.3	5.8	12.6	33.2
	0.5	3.1	6.0	12.8	35.0
	0.5	2.9	6.3	14.1	32.5
	0.6	3.1	5.9	12.2	35.2
Mean, mg/kg	0.2[b]	2.9[b]	5.4[b]	12.5[b]	33.5
SD, mg/kg	0.2	0.2	0.6	1.2	1.5

[a] Values are measured concentrations (milligrams per kilogram milk protein). Concentrations below 2.5 mg/kg milk protein were calculated using a second-order polynomial curve fitting. All other concentrations were calculated using RIDA SOFT Win.

[b] Significant differences between analysts ($P < 0.01$) by ANOVA.

Table 2. LOD calculation for cookies[a]

Cookies	Blank matrix	Spiked at 2.5 mg/kg	Spiked at 5 mg/kg	Spiked at 10 mg/kg	Spiked at 30 mg/kg
Analyst 1	−0.3	2.7	5.4	10.4	34.1
	−0.3	2.7	4.4	9.6	35.9
	−0.5	2.6	4.4	10.2	32.2
	−0.5	2.5	4.6	10.2	30.3
	−0.4	2.5	4.2	9.8	32.6
Analyst 2	−0.2	2.8	5.7	13.2	36.3
	−0.2	2.9	5.5	13.2	36.2
	−0.2	2.8	5.3	15.0	34.7
	−0.1	2.5	5.6	14.3	34.4
	0.0	2.4	5.9	14.1	36.5
Analyst 3	−0.3	3.0	6.2	12.5	27.5
	−0.4	3.1	4.9	12.8	29.5
	−0.3	3.0	5.2	14.3	28.5
	0.2	3.0	4.9	12.5	30.0
	0.0	3.1	5.0	7.9	31.8
Mean (mg/kg)	−0.2[b]	2.8[b]	5.1[b]	12.0[b]	32.7[b]
SD (mg/kg)	0.2	0.2	0.6	2.1	3.0

[a] Values are measured concentrations (milligrams per kilogram milk protein). Concentrations below 2.5 mg/kg milk protein were calculated using a second-order polynomial curve fitting. All other concentrations were calculated using RIDA SOFT Win.

[b] Significant differences between analysts ($P < 0.01$) by ANOVA.

This corresponds to an LOD with the probability of 5% of false positives and false negatives ($\alpha = \beta = 0.05$; corresponds to 1.65 times SD each). Accordingly, the LOD was calculated as 1.3 mg/kg for chocolate dessert and 0.8 mg/kg for cookies, resulting in a mean LOD of 1.0 mg/kg.

When using only the lower three concentrations closer to the LOD, the LOD is calculated as 0.8 mg/kg for chocolate dessert and 0.3 mg/kg for cookies, resulting in a mean LOD of 0.5 mg/kg. Also widely used is the calculation of the LOD solely on the basis of the mean value and SD of blank samples [LOD = mean (blank) + 3.3 × SD (blank)]. This calculation is also accepted by ref. 1 as the "basic formula." Using again the same data set as for the other calculations, the LOD is calculated as 0.9 mg/kg for chocolate dessert and 0.4 mg/kg for cookies, resulting in a mean LOD of 0.6 mg/kg (Table 3).

Because the sigmoid behavior of the data points is only poorly approximated by the linear regressions, and exact guidelines on how to calculate the LOD are missing, the mean value of all three different approaches described above was used for LOD estimation. This calculation resulted in an estimated LOD of 0.7 mg/kg milk protein (Table 3).

Table 3. Overview of LOD values according to different calculation models[a]

Matrix	Linear regression of five concentrations	Linear regression of the lowest three concentrations	SD of blank samples	Mean
Chocolate dessert	1.3	0.8	0.9	1.0
Cookies	0.8	0.3	0.4	0.5
Mean	1.0	0.5	0.6	0.7

[a] Values are in milligrams per kilogram milk protein).

LOQ

In general, an accurate quantification is only possible within the range of the calibrators, because only this range is approximated by the used mathematical function (cubic spline for RIDA SOFT Win). For all samples outside the calibrator range, the mathematical function has to be extrapolated, which increases uncertainty, i.e., for the RIDASCREEN FAST Milk test, the theoretically maximal range of quantification is from Calibrator 2 (2.5 mg/kg milk protein) to Calibrator 5 (67.5 mg/kg milk protein). Because samples with higher concentrations can simply be diluted to lie within the range of calibrators, the lower limit (LOQ) is a key characteristic of each test.

The LOQ can also be calculated according to ref. 1 with the formula LOQ = 3 × LOD. Using the mean value of the LOD calculation above, the LOQ equals 0.73 mg/kg × 3 = 2.19 mg/kg, confirming the set LOQ of 2.5 mg/kg.

To confirm the minimum theoretical value for the LOQ of 2.5 mg/kg milk protein, mean values and SDs of samples spiked with 2.5 mg/kg milk protein and of Calibrator 2 (used as normal sample) were calculated (five replicates each). Each calibrator and sample was tested in duplicate (Table 4). The P value of 0.79 was considered nonsignificant (higher than 0.1); thus, there is no significant difference between a real sample with 2.5 mg/kg milk protein and Calibrator 2. Accurate quantification is therefore achieved at a concentration of 2.5 mg/kg milk protein, and the LOQ is set to this concentration.

Table 4. LOQ calculation[a]

Matrix	Measured concentration, mg/kg	Mean, mg/kg	SD, mg/kg	P[b]
Chocolate dessert spiked at 2.5 mg/kg	2.8	2.7	0.1	0.79
	2.7			
	2.6			
	2.6			
	2.6			
Cookies spiked at 2.5 mg/kg	2.7	2.6	0.1	
	2.7			
	2.6			
	2.5			
	2.5			
Calibrator 2	2.7	2.7	0.2	
	2.5			
	2.5			
	2.8			
	2.8			

[a] Data from one analyst. Concentrations below 2.5 mg/kg milk protein were calculated using a second-order polynomial curve fitting. All other concentrations were calculated using RIDA SOFT Win.

[b] Calculated by ANOVA.

According to these data, the "real" LOQ is probably lower than 2.5 mg/kg milk protein, because the RIDASCREEN FAST Milk test most likely has the capacity to detect lower concentrations with sufficient accuracy.

Cross-Reactivity Testing

The compounds were treated like normal unknown samples, i.e., 1 g compound was extracted with 4 mL Extractor 2 for 10 min at 100°C/212°F, followed by addition of 16 mL A-AEP buffer and a second extraction step for 10 min at 60°C/140°F.

The tested compounds/commodities were almond, barley (milled), brazil nut, beef, buckwheat (milled), cashew, chickpea, cacao, corn flour, crustacean/prawn, whole egg powder, egg white powder, fish gelatin, fish (codfish), hazelnut, lecithin (soy), lima bean, oat (flakes), pea (milled), peanut, pecan nut, pistachio, poppy seed, rice flour, white rice (milled), brown rice (milled), rye (milled), sesame seed, sesame seed (roasted), soybean (milled), soy milk, soy protein, split pea tofu, walnut, wheat (milled). In addition, for pine nut, pumpkin seed, and sunflower seed, 0.5 g bovine serum albumin (fraction 5) was added to 1 g sample.

This extract was further diluted 1:5 with Allergen Extraction buffer. Thus, the final concentration in buffer that was added to the wells was 10 mg compound/mL extraction buffer, which corresponds to the level of a 100% sample. Each calibrator and sample was tested in duplicate (two wells per calibrator/sample). None of the matrixes investigated on a level of 10 mg/mL extraction buffer showed any cross-reactivity above the LOQ.

Ruggedness Study

The influence of varying incubation temperature, reagent volume, incubation time, extraction temperature, and extraction time on milk ELISA was investigated. Incubation temperature, reagent volume, and incubation time were applied to all steps of the milk ELISA. All test reagents and analyzed samples were equilibrated to the requested temperature for approximately 45 min prior to testing. Five replicates of rice flour containing approximately 5 mg/kg milk protein were extracted and analyzed according to the different conditions. The incurred sample was characterized by homogeneity testing to contain 5.6 ± 0.5 mg/kg milk protein. Each calibrator and sample was tested in duplicate (two wells per calibrator/sample). In detail, the following conditions were investigated:

Incubation time.—9, 10 (standard protocol), and 11 min.

Reagent volume.—90, 100 (standard protocol), and 110 µL.

Incubation temperature.—18, 23 (standard protocol), and 37°C.

Extraction time at 100°C.—9, 10 (standard protocol), and 11 min.

Extraction time at 60°C.—9, 10 (standard protocol), and 11 min.

Extraction temperature.—55, 60 (standard protocol), and 65°C.

Sample concentrations were calculated for each ruggedness condition in relation to the respective calibration curve using RIDA SOFT Win (Table 5). The three variations of one varied parameter were analyzed with ANOVA software to identify variations that led to a significant change in measured concentrations. No significant variation ($P < 0.01$) in measured concentrations was observed.

Table 5. Summary of ruggedness testing[a]

Ruggedness condition	Mean, mg/kg	SD, mg/kg	P^b
Standard procedure	5.6	0.4	
Incubation time, min			
9	6.2	0.6	0.34
11	5.9	0.9	
Reagent volume, µL			
90	5.5	0.5	0.94
110	5.5	0.5	
Incubation temperature, °C			
18	5.3	0.2	0.40
37	5.4	0.4	
Step 1 extraction time, min			
9	5.8	0.3	0.33
11	5.4	0.5	
Step 2 extraction time, min			
9	5.2	0.7	0.29
11	4.9	0.7	
Step 2 extraction temperature, °C			
55	5.4	0.5	0.75
65	5.3	0.7	

[a] Mean and SD values are calculated concentrations (milligrams per kilogram milk protein).

[b] Calculated by ANOVA for the three variations of one varied parameter (standard protocol and two variations).

Lot-to-Lot Stability Study

Three independent lots of the RIDASCREEN FAST Milk kit were tested to ensure consistent manufacturing between lots and to support the reported shelf life of the test kits. The shelf life is set according to the component with the shortest remaining shelf life. For the RIDASCREEN FAST Milk kit, the maximum shelf life is 18 months. The test documented by the QA department at R-Biopharm AG consisted of official product batch release testing by the QA department and additional real-time stability testing after regular storage intervals (usually approximately 3 months). The absorbances of calibrators and the concentrations of control samples were evaluated.

In the course of this validation, the absorbances of calibrators were investigated and the accuracy of the ELISA was tested with two negative samples and two positive samples containing approximately 10 mg/kg milk protein. All concentrations were calculated with RIDA SOFT Win.

A general loss of absorbance was observed for all calibrators. However, this loss did not affect the result of the test. All negative samples were found well below the LOQ in all three lots, usually with an absorbance similar to Calibrator 1.

The positive samples were measured, with a mean concentration (SD noted in parentheses) of 10.5 (1.4) and 12.1 (0.9) mg/kg in Lot 1; 11.9 (1.2) and 11.5 (1.1) mg/kg in Lot 2; and 12.2 (1.5) and 11.8 (1.5) mg/kg in Lot 3. Thus, very good lot-to-lot stability and consistency could be shown over a range of 18 months.

Matrix Study

To test the ability of the RIDASCREEN®FAST Milk test to quantify milk proteins in different food matrixes, a matrix study was conducted. Milk-free cookie, infant formula, chocolate dessert, ice cream, and sausage blank samples were spiked prior to extraction at levels of 0, 5, 10, and 30 mg/kg milk protein. Five replicates of each spiking level per matrix were extracted and analyzed, resulting in 100 samples in total. Each calibrator and sample was tested in duplicate (two wells per calibrator/sample). Concentration and recovery of all spiking levels was calculated using RIDA®SOFT Win (Table 6). All negative samples were detected well below the LOQ. Mean recoveries for spiked samples ranged between 94% (infant formula spiked with 30 mg/kg) and 128% (chocolate dessert spiked with 10 mg/kg). SDs ranged between 1.9% (ice cream spiked with 10 mg/kg) and 11.2% (cookies spiked with 10 mg/kg). The general elevation of the SD in cookies and infant formula is probably due to the high absorbance of liquids by the cookie material and infant formula powder. Spiking with a liquid solution might therefore be less reproducible in these matrixes than in less-absorbing matrixes such as ice cream and chocolate dessert. Overall, mean recovery was calculated as 105.1% and overall mean SD as 6.6%.

Table 6. Summary of matrix study

Spike level of matrix, mg/kg	Mean, mg/kg[a]	SD, mg/kg[a]	RSD, %	Mean recovery, %	SD recovery, %
Cookies					
Blank	<2.5				
5	4.9	0.5	9.7	99	9.6
10	11.6	1.1	9.6	116	11.2
30	33.6	3.2	9.6	112	10.8
Infant formula					
Blank	<2.5				
5	5.0	0.5	10.0	100	9.9
10	10.4	0.7	6.6	104	6.9
30	28.0	2.8	10.1	93	9.5
Chocolate dessert					
Blank	<2.5				
5	5.7	0.3	5.3	113	5.3
10	12.8	0.9	6.9	128	6.9
30	32.4	1.0	3.1	108	3.1
Ice cream					
Blank	<2.5				
5	4.9	0.2	4.4	97	4.3
10	11.2	0.2	1.7	112	1.9
30	30.8	0.9	2.9	103	2.9
Sausage					
Blank	<2.5				
5	4.7	0.4	7.4	94	6.9
10	10.2	0.7	6.7	102	6.8
30	29.1	1.1	3.6	97	3.5
Mean of matrixes spiked at 5 mg/kg	5.0	0.4	7.2	100	7.2
Mean of matrixes spiked at 10 mg/kg	11.2	0.8	6.7	112	6.7
Mean of matrixes spiked at 30 mg/kg	30.8	1.8	6.0	103	6.0
Overall mean[b]				105	6.6

[a] Values are calculated concentrations.

[b] Overall mean values were calculated from the mean values of each spiking level of each matrix (n = 5).

Table 7. Overview of preparation of cookies with four different levels of milk protein[a]

	Intended milk protein concentration level, mg/kg			
Component	0	5	10	30
Premix, g[b]	0	50	100	300
Blank wheat flour, g	300	250	200	0

[a] Preparation involved adding wheat semolina, sucrose, vanilla sugar, and salt to each of the four levels of Premix and Blank wheat flour and mixing thoroughly; adding egg yolk, margarine, and water to each level and mixing thoroughly using a handheld agitator; and incubating at 4°C overnight. The final dry mass of each level was approximately 670 g. Approximately 60 g cookies were formed, baked at 150°C for 15 min at upper and lower heat, cooled down to room temperature, dried at 60°C for 120 min, and then ground thoroughly.

[b] Premix = 450 g Wheat flour plus 86.9 mg MoniQA skim milk powder standard (32.25% milk protein).

Independent Validation Study

Analysis was conducted according to the methodologies outlined in the AOAC-RI *Performance Tested Methods* study protocol "The RIDASCREEN FAST Milk Enzyme Immunoassay for the Quantitative Analysis of Milk Protein" (August 28, 2013) in conjunction with the instructions for use included with the test kit.

Table 7 shows, in brief, the preparation of incurred cookie materials with different amounts of milk protein. Dried cookies were placed into a Robot-Coupe Blixer 4V food processor and ground thoroughly, and then 3 g portions of the ground cookie were removed, blind-coded, and stored at refrigeration (2–8°C) until the analysis was conducted. Table 8 depicts the preparation of chocolate dessert with different milk protein concentrations. The target levels prepared for the heat-processed cookies

Table 8. Overview of preparation of chocolate dessert with different levels of milk protein[a]

	Intended milk protein concentration level, mg/kg			
Component	0	5	10	30
Premix, g[b]	0	20	40	120
Blank starch/sucrose/cocoa, g	120	100	80	0

[a] Preparation involved adding corn oil and Tween to each of the four levels of Premix and Blank (consisting of starch/sucrose/cocoa powder) and mixing thoroughly. The final viscous mass of each level was 200 g.

[b] Premix = Thoroughly mixed combination of 39 g cold-swelling starch, 84 g sucrose, 57 g cocoa powder, and 25.5 mg MoniQA skim milk powder standard (32.25% milk protein).

Table 9. Independent validation study results for cookies[a]

Sample	OD[b]	RSD, %	Reported, mg/kg
1	0.095	8.9	<2.5
2	0.086	10.7	<2.5
3	0.085	26.6	<2.5
4	0.072	6.9	<2.5
5	0.090	17.3	<2.5

[a] Incurred cookie material target concentration: 0 mg/kg milk protein.

[b] OD = Optical density mean values for technical duplicates.

assume that all added water was removed by evaporation during the baking and drying process.

Results

For 0 mg/kg levels for dry cookies and the chocolate dessert, all samples tested returned values of <2.50 mg/kg (LOQ of the assay); therefore, no statistical analysis was conducted on these levels. Tables 9 and 10 present a summary of data for each matrix.

For the analysis of cookies and level combination, the mean, SD, and RSD were calculated. The SD results are presented in Table 11.

For the analysis of the chocolate dessert and level combination, the mean, SD, and RSD were calculated. The SD results are presented in Table 12.

Discussion

The RIDASCREEN FAST Milk test kit investigated in this validation proved highly applicable for the trace detection of milk proteins in a wide variety of foods. The in-house validation included the estimates of the LOD and LOQ, a cross-reactivity study, stability testing, a lot-to-lot comparison, a ruggedness study, and a matrix study. The validation by an independent laboratory included the analyses of incurred samples.

The LOD was calculated using different approaches that resulted in different LODs, although it was based on the same data set obtained from three different analysts using different equipment. The first approach described in ref. 1 is based on the measurement of SDs at different concentrations and plotting the SDs against the concentrations. This approach takes into account that the SD usually increases with increasing concentration. Using this approach with all five concentrations measured (0, 2.5, 5, 10, and 30 mg/kg), the LOD was calculated as 1.29 mg/kg for chocolate dessert and 0.78 mg/kg for cookies.

Table 10. Independent validation study results for chocolate dessert[a]

Sample	OD[b]	RSD, %	Reported, mg/kg
1	0.107	15.9	<2.5
2	0.114	5.0	<2.5
3	0.122	19.1	<2.5
4	0.133	1.6	<2.5
5	0.151	0.9	<2.5

[a] Incurred chocolate dessert target concentration: 0 mg/kg milk protein.

[b] OD = Optical density mean values for technical duplicates.

Table 11. Independent validation study results for incurred cookies

Target milk protein concn, mg/kg	Mean, mg/kg[a]	SD, mg/kg	RSD, %	Recovery, %
5	5.1	0.5	9.5	103
10	8.7	0.6	7.4	87
30	28.0	4.4	15.8	93

[a] Mean concentration values of 5 blinded individual samples.

However, the concentrations 10 and 30 mg/kg showed high influence on the linear regression (the second approach), although being far from the concentration of interest. Thus, the linear regression does not fit the calibration curve in the lower range of the curve, which is due to the sigmoid shape of the curve. Accordingly, linear regression of the three lower concentrations (0, 2.5, and 5 mg/kg) resulted in the much lower LOD of 0.75 mg/kg for chocolate dessert and 0.28 mg/kg for cookies. The third approach only uses the mean value and SD of blank matrix samples, resulting in an LOD of 0.89 mg/kg for chocolate dessert and 0.36 mg/kg for cookies. This approach is accepted in ref. 1 and referred to as the "basic formula."

To our knowledge, this is one of the first ELISA methods to use the first approach, i.e., using SDs at several concentrations to calculate the LOD. According to our data, it appears that the SD increases slowly at low concentrations. The SD at low concentrations might therefore be influenced mainly by the test setting itself (e.g., pipetting volume inaccuracy and photometer inaccuracy) rather than by concentration. This assumption is supported by the fact that chocolate dessert and cookies show almost exactly the same behavior for the three lower concentrations. However, higher concentrations lead to an increase of SD. Overall, the curve has a sigmoid shape, which is the reason for the poor approximation by linear regression. For future validations, it therefore seems advisable to use concentrations close to the putative LOD/LOQ and to omit high concentrations for calculation of the LOD when possible. Alternatively, the basic formula approach would also be a good calculation method for sigmoid curves that have a very low slope for low concentrations. Based on this validation, the LOD, estimated using the overall mean of all three approaches, is 0.73 mg/kg milk protein.

Independent of the LOD approach chosen, the method showed high accuracy and repeatability over the calibration range. The mean and SD of samples spiked with 2.5 mg/kg milk protein and of Calibrator 2 (concentration, 2.5 mg/kg milk protein) did not differ significantly. Samples at a concentration of 2.5 mg/kg milk protein can thus be accurately and precisely measured. The

Table 12. Independent validation study results for incurred chocolate dessert

Target milk protein concn, mg/kg	Mean, mg/kg[a]	SD, mg/kg	RSD, %	Recovery, %
5	4.9	0.5	10.0	98
10	10.4	0.2	2.3	104
30	31.5	2.0	6.5	105

[a] Mean concentration values of 5 blinded individual samples.

LOQ was therefore set to 2.5 mg/kg milk protein, corresponding to the calibrator with the lowest milk protein concentration.

The cross-reactivity study with 39 substances (grains, vegetables, seeds, nuts, and miscellaneous compounds) used as 100% samples showed that none of the substances had a cross-reactivity above the LOQ.

A thorough ruggedness testing included analyzing variations in test implementation and sample preparation. Variation of incubation temperature (18, 23, and 37°C), reagent volume (±10%), incubation time (±10%), extraction temperature (±5°C), and extraction time (±10%) did not show significant influence on the test results.

The stability testing of three independent lots showed high lot-to-lot reproducibility and high accuracy. A general decrease in the absorbances is expected and was also observed over the shelf life due to the loss of activity of components. However, this decrease is mainly due to loss of binding activity of the microtiter plate, binding activity of the conjugate, and the enzymatic activity of the conjugate. Calibrators and samples are therefore equally affected, leading to an unchanged concentration measurement over the shelf life. Accurate determination of concentrations could be shown in real-time stability testing.

The matrix study included five different matrixes (cookies, infant formula, chocolate dessert, ice cream, and sausage). Unspiked samples and spiked samples at three different concentrations were used. All blank samples were detected well below the LOQ. The spiked samples showed generally high accuracy (mean recovery, 105.1%) and repeatability (mean SD of recovery, 6.6%).

An independent evaluation of the RIDASCREEN FAST Milk test was performed by an independent laboratory and consisted of the analysis of incurred cookies and chocolate dessert. Both materials containing 0, 5, 10, and 30 mg/kg of milk protein were manufactured at the independent laboratory using standard food production protocols and tested with five replicates each. Recoveries ranged from 87% up to 103% (cookies) and 98% up to 105% (chocolate dessert). RSD values from 7.4–15.8% (cookies) and from 2.3–10.0% (chocolate dessert) were reported, showing good accuracy and repeatability for analysis of incurred samples. For the 0 mg/kg level for both materials, all samples tested returned values of <2.5 mg/kg (LOQ of the assay); thus, no statistical analysis was conducted on these levels. Therefore, the analytical performance claims of the manufacturer were confirmed.

In summary, the RIDASCREEN®FAST Milk test is applicable for the detection of milk proteins in a wide variety of foods and can therefore contribute to ensure a milk protein–free diet for predisposed individuals.

Conclusions

All data collected show that the RIDASCREEN®FAST Milk test is capable of analyzing milk proteins at concentrations from 2.5 to 30 mg/kg in processed cookies, chocolate, chocolate dessert, ice cream, infant formula, and sausages. The LOD of the method is 0.7 mg/kg. The test kit is robust against variation of the test implementation itself, as well as sample preparation.

The test kit manufacturer and the external laboratory recommend the method as an AOAC *Performance Tested Method*.

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