Analysis of Bovine Lactoferrin in Infant Formula and Adult Nutritional Products by Optical Biosensor Immunoassay: First Action 2021.07

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Abstract

Background: Bovine lactoferrin is increasingly being used as an ingredient in infant formula manufacture to enhance nutritional efficacy through the provision of immunoprotective, growth, and antimicrobial factors to the neonate.

Objective: To evaluate the analytical performance of an optical biosensor immunoassay for compliance with the method performance requirements described in SMPR 2020.005.

Methods: Following dilution of the sample in buffer, an automated, label-free, real-time optical biosensor immunoassay was used in a direct assay format to quantitate bovine lactoferrin by its interaction with an immobilized anti-lactoferrin antibody. Quantitation was accomplished by the external standard technique with interpolation from a 4-parameter calibration regression.

Results: The analytical range (0–200 mg/hg), method detection limit (0.8 mg/hg), recovery (96.1–109.2%), and repeatability (1.0–5.3%) complied with the requirements given in the lactoferrin SMPR. The method was shown to be specific for native, intact lactoferrin; thermally denatured lactoferrin generated no measurable binding response.

Conclusions: The method described is suitable for the quantification of intact, undenatured lactoferrin in milk products, infant formulas (bovine milk protein-based, soy protein-based, and amino acid-based), and adult nutritionals and has been demonstrated to meet the performance requirements defined in SMPR 2020.005.

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Highlights: A single-laboratory validation (SLV) of an automated biosensor immunoassay for the determination of intact, undenatured lactoferrin is described.

Introduction

Milk contains numerous minor proteins with physiological properties that are targeted at providing immunoprotective, growth, and antimicrobial factors to the neonate, as distinct from the nutritionally more significant major proteins. Lactoferrin is an approximately 80 kDa basic (pI: approximately 9.0), iron-binding, bi-lobal, secretory transport, sialylated glycoprotein of known amino acid sequence and is a member of the transferrin family, which is characterized by carbonate-aniondependent, high-affinity, and reversible binding of two Fe^{3+} ions per molecule (1). In addition to its antimicrobial activity, lactoferrin may function in intestinal iron uptake and regulation, immune response, growth factor activity, bone growth, and antioxidant activity (2). The lactoferrin content is species dependent, with significantly higher levels in human milk and colostrum than in the bovine equivalents, which has stimulated an increasing trend of the supplementation of bovine milk-based infant formulas with fractionated bovine lactoferrin, despite a 69% primary sequence homology with human lactoferrin (3).

The increasing commercial interest in exploiting the therapeutic value of lactoferrin has stimulated the need for reliable concentration assays for its determination at endogenous levels in milk and colostrum, at supplemental levels in infant formulas, and at pharmaceutical levels in milk protein isolates. Several chromatographic techniques have been reported, including recent LC–tandem MS strategies to address this challenge (4–6). Alternative immunoassay techniques are also available and, compared with separation-based techniques, offer the advantages of a simpler sample preparation and estimation of the intact physiologically active concentration of the protein. A comprehensive review of current analytical methods for lactoferrin has been recently reported (7). A label free, real-time, automated optical biosensor immunoassay for the quantitation of intact, undenatured lactoferrin in milk, colostrum, and lactoferrin-supplemented infant formulas has been routinely implemented within the New Zealand dairy industry for approximately 15 years (8).

This SLV study assesses the performance of the method (calibration, detection limit, specificity, precision, and recovery) against the Standard Method Performance Requirements (SMPR) for lactoferrin recently established by the Stakeholder Program on Infant Formula and Adult Nutritionals (SPIFAN).

AOAC Official Method 2021.07

Analysis of Bovine Lactoferrin in Infant

Formula and Adult Nutritional Products

Optical Biosensor Immunoassay

First Action 2021

[Applicable to the determination of bovine lactoferrin in bovine-milk protein-based, soy protein-based, and amino acid based, infant formula, and adult nutritional powders.]

Caution: Refer to the Material Safety Data Sheets for all chemicals prior to use. Use all appropriate personal protective equipment and follow good laboratory practices.

A. Principle

The bovine lactoferrin content in infant, adult, and/or pediatric formulas is determined by an automated biosensor-surface plasmon resonance (SPR)-based immunoassay using immobilized goat anti-bovine lactoferrin antibody as the detecting molecule. The method is configured as a direct and non-labelled immunoassay, with quantitation against an authentic bovine lactoferrin calibrant. The sample is prepared for analysis by simple dilution into buffer.

B. Apparatus

a) Automated biosensor instrument.—BiacoreVR T200 or Biacore Q (GE Healthcare, Uppsala, Sweden) or equivalent SPR-based biosensor.
b) Sensor chip.—CM5 (GE Healthcare) or equivalent.
c) Micropipettes.—10–100 mL, 100–1000 mL, and 1–10 mL.
d) Centrifuge tubes.—15 mL, polypropylene.
e) Volumetric flasks.—5, 10, 50, and 100 mL.
f) Microtiter plates.—96-well, polystyrene.
g) Microcentrifuge tubes.—1.5 mL, polystyrene.
h) Balance.—Accurate to 4 decimal places.

C. Reagents

a) Antibody.—Affinity-purified, polyclonal goat anti-bovine lactoferrin, 1 mg/mL (A10-126A, Bethyl Laboratories, Montgomery, TX, USA or equivalent).
b) Bovine lactoferrin.—Approximately 50 mg (certified reference material, Cerilliant, Round Rock, TX, USA or equivalent).
c) Amine coupling kit.—GE Healthcare or equivalent individual commercially available reagents.
   (1) EDC.—0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl.
   (2) NHS.—0.1 M N-hydroxysuccinimide.

(3) Ethanolamine.—1 M, pH 8.5.

d) Immobilization buffer.—Sodium acetate, 10 mM, pH 5.0 (GE Healthcare/Cytiva, Marlborough, MA, USA or equivalent).

e) Running buffer (HPS-EP).—10 mM HEPES, 0.15 M NaCl, 3.0 mM EDTA, 0.005% surfactant P20, pH 7.4 (GE Healthcare or equivalent).

f) Glycine-HCl.—10 mM, pH 1.5 and pH 2.0 (GE Healthcare or equivalent).

g) Hydrochloric acid.—0.1 M (Merck, Darmstadt, Germany or equivalent).

h) Sodium chloride.—AR grade (Sigma, St. Louis, MO, USA or equivalent).

i) Water.—Purified with resistivity ≥ 18 MΩ.

j) HPLC vials, septa, and caps.

D. Reagent Preparation

(a) Solution buffer.—Dissolve 2.044 g sodium chloride in 100 mL HPS-EP. Store in a refrigerator at 4 °C. Expiry: 3 months.

(b) Regeneration reagent (10 mM glycine-HCl, pH 1.75).—Prepare a mixture of 10 mM glycine pH 1.5—10 mM glycine pH 2.0 (36 + 64, by volume). Prepare fresh.

E. Immobilization

a) Immobilization of anti-bovine lactoferrin antibody to a CM5 sensor surface is via amine coupling.

(b) A designated flow cell is activated with 0.4 M EDC–0.1 M NHS (1 + 1, by volume, 10 mL/min, 7 min), followed sequentially with the injection (10 mL/min, 7 min) of goat anti-bovine lactoferrin (50 mg/mL in 10 mM sodium acetate, pH 5.0).

(c) Unreacted ester functionalities are deactivated with ethanolamine (1 M, pH 8.5, 10 mL/min, 7 min).

(d) Final immobilization levels in resonance units (RU; where 1 RU = 1 pg/mm^2) are determined from the sensorgram and are typically approximately 10–15 kRU. The chip may be stored between analyses over desiccant at 4 °C in a sealed container.

F. Standard Preparation

(a) Stock standard (SS).—Approximately 5 mg/mL corrected for purity from Certificate of Analysis.

(1) Weigh accurately approximately 50 mg of lactoferrin and dissolve in 10 mL of water.

(2) Store in a freezer under nitrogen at less than < 10 °C until required.

(b) Intermediate standard (IS).—100 µg/mL.

(1) Dilute SS to exactly 100 µg/mL by appropriate dilution in HPS-EP.

(2) Sub-aliquots may be stored in a freezer under nitrogen at less than < 10 °C until required.

(c) Working standards (WS).

(1) To 990 µL solution buffer in a plastic vial, add 10 µL IS. This is WS1 (1000 ng/mL). Prepare fresh each run.

(2) Prepare a calibration standard series by 2-fold serial dilution. Label five plastic vials WS2–WS6. Pipette 500 µL solution buffer into each vial. Prepare fresh each run.

(a) WS2 = 500 ng/mL, pipette 500 µL WS1 into vial 2; cap and vortex.

(b) WS3 = 250 ng/mL, pipette 500 µL WS2 into vial 3; cap and vortex.

(c) WS4 = 125 ng/mL, pipette 500 µL WS3 into vial 4; cap and vortex.

(d) WS5 = 62.5 ng/mL, pipette 500 µL WS4 into vial 5; cap and vortex.

(e) WS6 = 0 ng/mL, pipette 500 µL solution buffer only into vial 6; cap and vortex.

F. Sample Preparation

(a) Infant formula powder or milk powder (diluted 1:2000 w/v)

(1) Accurately weigh approximately 0.50 g of powder into a 15 mL centrifuge tube; record the weight.

(2) Dissolve in 8 mL HPS-EP and mix by vortexing. Make up to 10 mL with HPS-EP. Store in the dark for ≥ 15 min.

(3) Pipette 990 µL solution buffer into a microcentrifuge tube; add 10 µL of the diluted sample. Cap and vortex.

(b) Powder reconstituted ready-to-feed basis (diluted 1:2000 w/v)

(1) Accurately weigh approximately 25 g powder into a container; record the weight.

(2) Tare the balance and accurately weigh approximately 200 g water into the same container; record the weight.

(3) Warm to 25 °C for 10 min; mix the sample solution thoroughly to ensure complete dissolution.

(4) Weigh a 4.5 g aliquot of sample solution in a centrifuge tube; make up to 10 mL with HPS-EP; cap and vortex.

(5) Pipette 990 µL solution buffer into a microcentrifuge tube; add 10 µL of the diluted sample. Cap and vortex.

H. SPR Analysis

(a) Calibration standards and sample extracts (200 mL) are dispensed (in duplicate) into the appropriate wells of a 96-well microtiter plate and sealed with adhesive foil.

(b) Include in each run:

  (1) sample blank;

  (2) repeat check standard (WS2);

  (3) duplicate in-house infant formula QC sample.

(c) The instrument system and docked sensor chip is equilibrated and analysis is initiated under optimized conditions. Note: automated analysis with Biacore T200 may be performed via the concentration assay wizard or method builder options. The former is simpler, whereas the latter gives more flexibility with respect to flow path, reference subtraction, and assignment of report points. If an alternative vendor SPR system is used, follow the manufacturer's instructions for use of the software system for quantitative analysis.

(d) The HPS-EP flow rate is 10 mL/min and the contact time 300 s.

(e) For regeneration, 10 mM glycine-HCl, pH 1.75 at a flow rate of 50 mL/min with contact time 32 s is used.

(f) The response at 10 s after commencement of the dissociation phase, relative to the baseline sampled 10 s before sample injection, is used for quantitation.

(g) Each injection cycle requires approximately 15 min, with a complete 96-well microtiter plate completed in approximately 24 h, including system equilibration and duplicate multilevel calibration.

I. System Suitability Test

(a) Perform a surface performance test to stabilize the chip.

(b) The system suitability test is performed: (i) when a sensor chip has been freshly immobilized with an antibody, and (ii) at the beginning of every analytical run.

  (1) Run: 3 × lactoferrin standard WS1, 1000 ng/mL

  (2) The repeatability of the binding response should be < 5%.

  (3) The residual limits of the calibration curve should be < 5%.

J. Characterization of a New Antibody

The performance of an alternative commercially available candidate affinity-purified and non-labelled polyclonal antibody should be compared relative to the prescribed antibody (Bethyl Laboratories, affinity-purified, polyclonal goat anti-bovine lactoferrin), under direct assay conditions.

(a) Using the concentration of the antibody provided by the vendor, dilute the antibody in 10 mM sodium acetate, pH 5.0 prior to coupling to the sensor chip surface to achieve comparable immobilization levels (10–15 kRU).

(b) Multiple sensor chip flow cells may be covalently immobilized with different candidate antibodies using standard amine-coupling chemistry, and with lactoferrin calibrant subsequently injected over all surfaces under direct assay conditions to compare antibody performance (additionally, the assay may be reversed, with lactoferrin immobilized and multiple candidate antibodies injected sequentially as analyte).

(c) Binding responses with the top-level lactoferrin calibration standard should be confirmed to be comparable under recommended direct assay operating conditions. In addition, a 4-parameter regression curve over the prescribed calibrant range should yield comparable values for curve parameters (R_{hi}, Rlo, A_1, and A_2) and detection limits.

(d) The S/N and dynamic range should be comparable under recommended direct assay operating conditions.

(e) Confirm specificity and selectivity of the immobilized antibody by estimating minimal or negligible cross-reactivity (B_{50}) to other major milk proteins relative to bovine lactoferrin.

(f) A competitive inhibition experiment may also be performed to confirm the specificity of the tethered antibody for bovine lactoferrin. This is accomplished by evaluating the extent of binding inhibition of lactoferrin to the immobilized antibody after incubating with excess antibody in solution.

(g) Confirm that the sensor surface regeneration protocol is effective for the alternative antibody.

(h) Confirm the stability of the immobilized alternative antibody surface over replicate cycles.

(i) Confirm comparable precision estimates (repeatability and intermediate precision) as described in System Suitability Test.

K. Calculations

Calibration standards are run at the beginning and end of each sequence of samples to ensure minimal response drift across the analytical run. The calibration trend feature can be implemented to account for minimal drift across a sequence. The calibration curve is constructed using the 4-parameter polynomial regression given in Equation 1.

$$y = R_{hi} - \left(\frac{(R_{hi} - R_{lo})}{1 + \left(\frac{Lf_{conc}}{A1} \right)^{A2}} \right) \quad (1)$$

where:

y = instrument response (RU); R_{hi} = response at infinite concentration; R_{lo} = response at zero concentration; A_1 B_{50} (concentration at 50% binding saturation); A_2 = slope factor; and Lf_{conc} = concentration of lactoferrin (ng/mL).

For long sample sequences, a further set of calibration standards can be included within the sequence. The result file is processed within T200 evaluation software (or alternative SPR system), where both the calibration curve and raw data interpolation are automated as part of instrument operation.

K. Data Handling

Report data to the nearest µg/mL (liquids), µg/g (powders).

Results and Discussion

Optical biosensors utilizing SPR detection are an important evanescent field technique for the label-free and real-time monitoring of biomolecular interactions. Although they are predominantly used for evaluating kinetic and thermodynamic characteristics in drug discovery and life science environments, they have also found application within the food sector to facilitate concentration analysis. The described method measures the lactoferrin present in the soluble aqueous fraction of the sample types as defined. The analytical technique is based on an automated, biosensor platform that incorporates an SPR optical detection technique to detect the interaction between the immobilized, biospecific recognition antibody and lactoferrin in solution. This method is rapid, sensitive, precise, and accurate, and provides analytical information of the physiologically active protein content. Further, it is simple to implement and, because of its inherent antibody-based biospecific detection strategy, allows a facile sample preparation involving direct dilution in buffer.

Characterization of a New Antibody

Antibodies (polyclonal, monoclonal, and recombinant) are widely used for applications including flow cytometry, immunohistochemistry, western blotting, enzyme-linked immunosorbent assay, and biosensor immunoassays. It is challenging to consistently generate a high-performing antibody because: (i) some proteins do not elicit a strong immune response, (ii) other proteins are too immunogenic, and (iii) other proteins share similar homology with non-target proteins, yielding a less specific antibody. Commercially available antibodies require proper validation and QC to ensure that they are fit for purpose (9).

There is currently a lack of enforced standards for antibody validation and journals have historically not provided guidelines for publishing validation data for antibody-based concentration assays. Once a commercially available antibody has been selected based on appropriate host species, target protein

species, and vendor-reported cross-reactivity, western blot data, additives, etc., it is important to further validate the antibody post-purchase prior to routine use. It is recommended that high antibody levels be immobilized on the sensor surface to: (i) facilitate measurable responses from low lactoferrin concentrations, and (ii) favor mass-transport-limited binding.

Single-Laboratory Validation

A number of infant formulas provided in the SPIFAN kit were used in the evaluation of this method (Table 1). Because the SPIFAN kit contains no lactoferrin-fortified products and each matrix contains very low levels of lactoferrin, and for one sample no protein at all, these matrixes were spiked with lactoferrin at concentrations typical of those in fortified infant formula products.

In common with immunoassay techniques, a 4-parameter curve fit adequately described the dose–response relationship over the working calibration range. A 6-level calibration curve (in duplicate) was constructed for each experiment over the working calibration range of 0–1000 ng/mL. This yielded an analytical range of 0–200 mg/hg for both liquid and powder infant formula and adult nutritional samples and complied with the analytical range requirements of 4–200 mg/hg, as described in SMPR 2020.005 (10). Superimposed sensorgrams and calibration curves from lactoferrin standard solutions run over three different days are illustrated in Figures 1 and 2.

Precision was assessed for all samples by testing duplicate samples on three separate days by two analysts on a single instrument, with fresh calibration standards and reagents made each day. Repeatability as RSD for the method in the samples ranged between 1.0 and 5.3% RSD (Table 2), which complied with the ≤ 6% limit specified in SMPR 2020.005 (9). The HorRat values were within or better than the expected range of 0.3–1.3 (11). Intermediate precision ranged between 2.0 and 5.7% with a mean value of 3.8%. Precision was also estimated from multiple analyses of independent duplicates (n = 125 pairs) of the infant formula powder used as a QC sample on separate days between 2018 and 2020, by many different analysts and over multiple immobilized chip surfaces. Repeatability was calculated as 4.2% (HorRat = 0.4) and complied with the requirement of < 6% in SMPR 2020.005 (9). Intermediate precision for the infant formula QC powder was estimated from these data as 8.6%.

The LOQ, determined as the method detection limit, is defined as the minimum measured concentration of a substance that can be reported with 99% confidence that the measured concentration is distinguishable from the method blank results (12).

The LOQ was estimated from replicate analysis (n = 7) of a fluid milk sample and gave a standard deviation of 0.255 mg/hg on a reconstituted milk basis. The LOD and LOQ were calculated as 0.8 mg/hg and 2.5 mg/hg, respectively, which complied with the maximum limit of 4 mg/hg in SMPR 2020.005 (10).

Recovery was evaluated using unfortified samples within the SPIFAN kit. Each matrix was spiked with authentic lactoferrin standard at two levels: 50% (approximately 357 mg/hg) and 100% (714 mg/hg) of typical lactoferrin-fortified infant formula concentrations. Spiked and unspiked samples were analyzed on three separate days. The recoveries measured for lactoferrin were between 96.1 and 109.2% (Table 3), which were within the limits of 90–110% set in SMPR 2020.005 (10).

The lack of a certified reference material or a reference method precludes an estimation of bias for lactoferrin in infant formula by this biosensor method. However, as an indication of method accuracy, the concentration of endogenous lactoferrin in a fluid milk sample was determined by the described method to be 18 mg/dL, a value that is consistent with levels typically reported for fluid bovine milk. The concentration of lactoferrin in a lactoferrin-supplemented infant formula powder was estimated to be 72 mg/hg, a value that is consistent with the target value of 67 mg/hg (product specification range of 34–100 mg/hg).

The acceptable performance figures-of-merit obtained for all samples used in this study support the method scope including bovine milk-based formulas as well as soy-based and enteral-based formulations.

The described immunoassay is specific for the detection and quantitation of intact, undenatured bovine lactoferrin. This specificity is based on the biospecific recognition of bovine lactoferrin by the polyclonal antibody that was raised to the intact, undenatured antigen, and therefore yields an estimate of the concentration of physiologically active protein. As for any immunoassay technique, the potentials for non-specific binding and/or cross reactivity were evaluated and were confirmed to be of negligible analytical significance under the conditions described (8).

To demonstrate that this method did not detect denatured lactoferrin, a lactoferrin standard (7560 ng/mL) in 50 mM sodium bicarbonate (pH 8.5) was heated to 90 °C for 60 min. A 50 mL aliquot of this solution was added to sample extracts (approximately 75 mg/hg in powder sample) and was analyzed by the method. As a control, the same concentration of native, undenatured lactoferrin was added to sample extracts and analyzed by the method. The recovery of denatured lactoferrin was calculated as described in Equation 2.

$$\text{Recovery}(\%) = \frac{C_{DL} - C_U}{C_{NL} - C_{U\times 100}} \qquad (2)$$

where C_{DL} = concentration of denatured lactoferrin in the spiked sample; C_U = concentration of unspiked sample; and C_{NL} = concentration of native lactoferrin in the spiked sample.

The measurement of denatured lactoferrin as a percentage of intact lactoferrin ranged from -3.1 to 0.1%. These results indicate that thermally denatured lactoferrin was not detected by the method. Loss of the

conformational integrity of native lactoferrin after heat treatment and the resulting lack of recognition by the immobilized polyclonal antibody has previously been reported (13).

Given the phylogenetic distance between the host animal for antibody production (goat) and the antigen species (cow), it is highly unlikely that the antibody reagent generated in goat will recognize goat lactoferrin, and hence the method is restricted to measurement of bovine lactoferrin in bovine milk-based products only.

Conclusions

The method described is suitable for the quantitation of intact, undenatured bovine lactoferrin in bovine milk products, infant formulas (bovine milk protein-based, soy protein-based, and amino acid-based), and adult nutritionals, and has been demonstrated to meet the performance requirements defined in AOAC SMPR 2020.005.

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Conflict of Interest

The authors declares that they have no conflict of interest.

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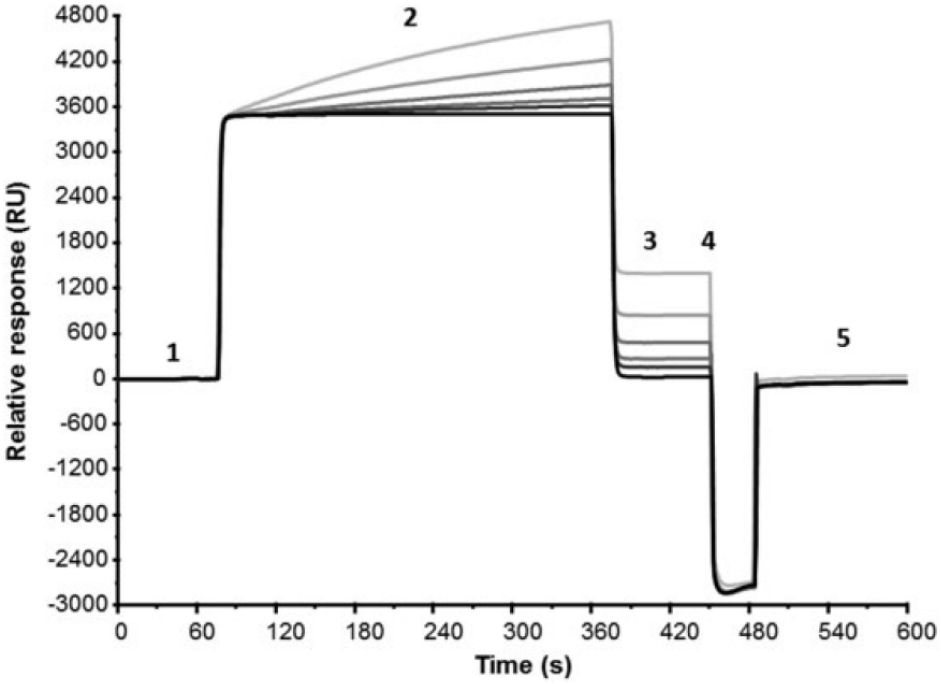

Figure 1. Superimposed lactoferrin calibration standard sensorgrams: (1) baseline, (2) association, (3) binding level, (4) regeneration, (5) baseline re-equilibration

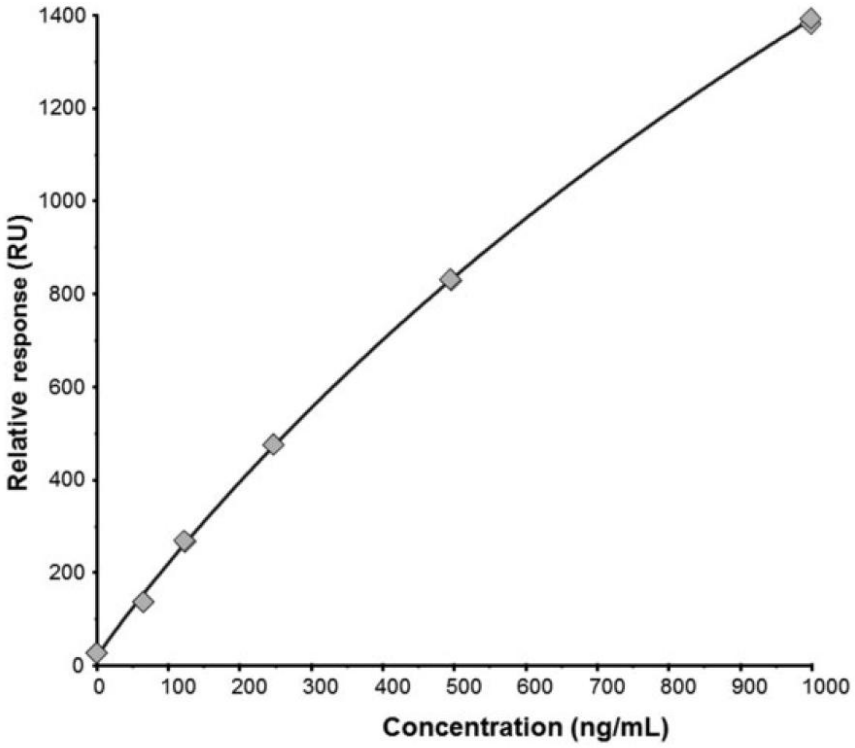

Figure 2. Superimposed 4-parameter calibration curves (three different days)

Table 1. SPIFAN kit samples used during method validation

Sample	Code	Lactoferrin fortified
Infant formula powder, milk-based	CULF358	No
Infant formula powder, soy-based	TJHR217	No
Infant formula powder, p/h-milk-baseda	KDOX966	No
Child formula powder, milk-based	RQXQ518	No
Adult nutritional powder, low-fat-milk-based	LYNY751	No
Infant formula powder, elemental-based	ECHL425	No
In-house QC infant formula powder, milk-based	—	Yes

[a] p/h = Partially hydrolyzed

Table 2. Repeatability and intermediate precision of method for lactoferrin

Sample	Repeatability RSD% (HorRat)	Intermediate Precision RSD%
Infant formula powder, milk-based	3.1 (0.3)	3.1
Infant formula powder, soy-based	5.2 (0.5)	4.3
Infant formula powder, p/h-milk-based	1.0 (0.1)	2.0
Child formula powder, milk-based	1.1 (0.1)	2.8
Adult nutritional powder, low-fat-milk-based	5.3 (0.5)	5.1
Infant formula powder, elemental-based	2.8 (0.2)	5.7

[a] p/h = Partially hydrolyzed

Table 3. Lactoferrin recovery of spiked samples

Sample	Mean Recovery, % (SD)	
	50% spike	Lactoferrin fortified
Infant formula powder, milk-based	98.5 (8.5)	101.8 (3.5)
Infant formula powder, soy-based	98.4 (5.1)	109.2 (3.0)
Infant formula powder, p/h-milk-based	102.9 (4.8)	98.6 (3.0)
Child formula powder, milk-based	100.5 (2.3)	101.5 (4.2)
Adult nutritional powder, low-fat-milk-based	97.9 (2.3)	104.5 (3.2)
Infant formula powder, elemental-based	96.1 (5.6)	104.2 (6.7)

[a] p/h = Partially hydrolyzed