ELISA Kit for Measuring Bovine Lactoferrin

CircuLex Bovine Lactoferrin ELISA Kit

Cat# CY-8098

Intended Use

The MBL Research Product **CircuLex Bovine Lactoferrin ELISA Kit** is used for the quantitative measurement of bovine lactoferrin in milk.

Individual users should determine appropriate conditions when using other types of samples.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store all components at 4°C.
- Don't expose reagents to excessive light.

Introduction

Lactoferrin is an iron-binding glycoprotein of the transferrin family that is expressed in most biologic fluids and is a major component of mammals' innate immune system (1). It is a glycoprotein consisting of a single polypeptide chain of about 80 kDa with two globular lobes each containing an iron-binding site. It is found in most exocrine secretions including milk, particularly the colostrums, and other secretions, such as tears, saliva, intestinal mucus and genital secretions, and in the specific granules of neutrophils.

The physiological roles that have been proposed for lactoferrin include anti-inflammatory, immunomodulatory, antimicrobial, antiviral and antitumoral functions. For this reason, lactoferrin is regarded as a host-defense mediator. Specific lactoferrin receptors exist in a variety of cells, including monocytes, lymphocytes, adipocytes, hepatocytes, and endothelial cells (2). Therefore determination of the lactoferrin concentration in various body fluids can be a marker of inflammation (3-6). It was reported that fecal lactoferrin is useful as a sensitive and specific marker in identifying intestinal inflammation such as Crohn's disease and chronic inflammatory bowel disease (IBD) in human (7). Combination of several markers, such as S100A8/A9 complex, defensin, elastase, MPO and I-FABP may be useful for classifying IBD.

Dietary supplements of bovine lactoferrin are purported in consumer literature to enhance and support the immune system response through their antioxidant, antibacterial, and antiviral properties. Several scientific researches revealed that oral supplements of bovine lactoferrin may be a useful adjunct toward modulation of immune activity, in particular T-cell activation and antioxidant status (8).

Principle of the Assay

The MBL Research Product **CircuLex Bovine Lactoferrin ELISA Kit** employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for bovine lactoferrin is pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any bovine lactoferrin present. After washing away any unbound substances, an HRP conjugated polyclonal antibody specific for bovine lactoferrin is added to the wells. Following a wash to remove any unbound antibody HRP conjugate, the remaining conjugate is allowed to react with the substrate H_2O_2-tetramethylbenzidine. The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance is proportional to the concentration of bovine lactoferrin. A standard curve is constructed by plotting absorbance values versus bovine lactoferrin concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

Summary of Procedure

Add 100 µL of diluted samples to the wells
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 100 µL of HRP conjugated anti- bovine lactoferrin antibody
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 100 µL of Substrate Reagent
↓ Incubate for 10-20 minutes at room temp.
Add 100 µL of Stop Solution
↓
Measure absorbance at 450 nm

Materials Provided

All samples and standards should be assayed in duplicate. The following components are supplied and are sufficient for the one 96-well microplate kit.

Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a foil, zip-lock bag with a desiccant pack. Wells are coated with anti-bovine lactoferrin monoclonal antibody (AF-3F8) as a capture antibody.

10X Wash Buffer: One bottle containing 100 mL of 10X buffer containing Tween®-20

Dilution Buffer: One bottle containing 50 mL of 1X buffer; use for reconstitution of bovine Lactoferrin Standard and sample dilution. Ready to use.

Bovine Lactoferrin Standard: One vial each containing X* ng of lyophilized bovine lactoferrin.
***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

HRP conjugated Detection Antibody: One bottle containing 12 mL of HRP (horseradish peroxidase) conjugated anti-bovine lactoferrin antibody. Ready to use.

Substrate Reagent: One bottle containing 20 mL of the chromogenic substrate, tetra-methylbenzidine (TMB). Ready to use.

Stop Solution: One bottle containing 20 mL of 1 N H_2SO_4. Ready to use.

Materials Required but not Provided

- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 µL precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **Orbital microplate shaker**
- **Microcentrifuge and tubes** for sample preparation.
- **Vortex mixer**
- **(Optional) Microplate washer:** Manual washing is possible but not preferable.
- **Plate reader** capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.
- **(Optional) Software package facilitating data generation and analysis**
- **500 or 1,000 mL graduated cylinder**.
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

Precautions and Recommendations

- **Although we suggest to conduct experiments as outlined below, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.**
- Allow all the components to come to room temperature before use.
- All microplate strips that are not immediately required should be returned to the zip-lock pouch, which must be carefully resealed to avoid moisture absorption.
- Do not use kit components beyond the indicated kit expiration date.
- Use only the microtiter wells provided with the kit.
- Do not use polystyrene tubes or sample plates for preparation or dilution of the samples.
- Rinse all detergent residues from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents in this kit may contain preservatives or other chemicals. Care should be taken to avoid direct contact with these reagents.
- Do not mouth pipette or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide.
- Wear gloves and eye protection when handling immunodiagnostic materials and samples of bovine origin, and these reagents. In case of contact with the Stop Solution and the Substrate Solution, wash skin thoroughly with water and seek medical attention, when necessary.
- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**
- **CAUTION: Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**

Sample Collection, Storage and Dilution

Sample Preparation

Milk, colostrum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference. For all samples, clarify by centrifugation and/or filtration prior to dilution in Dilution Buffer. If samples will not be assayed immediately, stored refrigerated for up to a few days, or frozen for long-term storage.

Other biological samples: MBL has not tested.
(*e.g.* Remove any particulates by centrifugation and assay immediately or aliquot and store samples at below -70°C. Avoid repeated freeze-thaw cycles. Individual users should determine appropriate conditions when using other types of samples.)

Sample Dilution

Dilute the samples with **Dilution Buffer**, based on the expected concentration of bovine lactoferrin, to fall within the range of the standard curve. For example, bovine milk samples may need to be diluted around 1:5,000.

• Remarks regarding recommended sample dilution
*For accurate quantification of samples that yield results outside the range of the standard curve, a lesser or greater dilution might be required. If unsure of sample level, a serial dilution with one or two representative samples before running the entire plate is highly recommended.
*The recommend dilution for samples should be used as a guideline. The recovery of bovine lactoferrin from an undiluted sample is not 100% and may vary from sample to sample. When testing less diluted samples it is advisable to run recovery experiments to determine the influence of the matrix on the detection of bovine lactoferrin

Detailed Protocol

The MBL Research Product **CircuLex Bovine Lactoferrin ELISA Kit** is provided with removable strips of wells so the assay can be carried out on separate occasions using only the number of strips required for the particular determination. Since experimental conditions may vary, an aliquot of the bovine Lactoferrin Standard within the kit should be included in each assay as a calibrator. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Preparation of Working Solutions

All reagents need to be brought to room temperature prior to the assay. Assay reagents are supplied ready-to-use, with the exception of **10X Wash Buffer** and **Bovine Lactoferrin Standard**.

1. Prepare a working solution of Wash Buffer by adding 100 mL of the **10X Wash Buffer** to 900 mL of deionized (distilled) water. Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Reconstitute **Bovine Lactoferrin Standard** with X* **mL** of **Dilution Buffer**. The concentration of the bovine lactoferrin in vial should be **200 ng/mL**, which is referred to as a **Master Standard** of bovine lactoferrin.
   ***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

   Prepare Standard Solutions as follows:
   Use the **Master Standard** to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 50 ng/mL standard (Std.1) serves as the highest standard. The **Dilution Buffer** serves as the zero standard (Blank).

	Volume of Standard	Dilution Buffer	Concentration
Std.1	150 µL of Master Standard (200 ng/mL)	450 µL	50 ng/mL
Std.2	300 µL of Std. 1 (50 ng/mL)	300 µL	25 ng/mL
Std.3	300 µL of Std. 2 (25 ng/mL)	300 µL	12.5 ng/mL
Std.4	300 µL of Std. 3 (12.5 ng/mL)	300 µL	6.25 ng/mL
Std.5	300 µL of Std. 4 (6.25 ng/mL)	300 µL	3.13 ng/mL
Std.6	300 µL of Std. 5 (3.13 ng/mL)	300 µL	1.56 ng/mL
Std.7	300 µL of Std. 6 (1.56 ng/mL)	300 µL	0.78 ng/mL
Blank	-	300 µL	0 ng/mL

Note: Do not use a Repeating pipette. Change tips for every dilution. Wet tip with Dilution Buffer before dispensing. Unused portions of Master Standard should be aliquoted and stored at below -70°C immediately. Avoid multiple freeze and thaw cycles.

Assay Procedure

1. Remove the appropriate number of microtiter wells from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.

2. Dilute samples with **Dilution Buffer**. (See the section "Sample Collection, Storage and Dilution" above.)

3. Pipette **100 µL** of **Standard Solutions (Std1-Std7, Blank)** and **diluted samples** in duplicates, into the appropriate wells.

   Note: For ease of loading samples, it is recommended that a second uncoated microplate should be used keeping diluted samples. This enables samples to be transferred quickly to the ELISA plate using multichannel pipette

4. Incubate the plate **at room temperature (ca.25°C) for 60 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker.

5. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

6. Add **100 µL** of **HRP conjugated Detection Antibody** into each well.

7. Incubate the plate **at room temperature (ca.25°C) for 60 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker.

8. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

9. Add **100 µL** of **Substrate Reagent**. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminum foil is recommended. Return Substrate Reagent to 4°C immediately after the necessary volume is removed

10. Incubate the plate **at room temperature (ca.25°C) for 10-20 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker. The incubation time may be extended up to 30 minutes if the reaction temperature is below than 20°C.

11. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

12. Measure absorbance in each well using a spectrophotometric microplate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the microplate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Note-1: Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
Note-2: Reliable standard curves are obtained when either O.D. values do not exceed 0.25 units for the blank (zero concentration), or 3.0 units for the highest standard concentration.
Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the

highest standard, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine bovine lactoferrin concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

Calculations

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation. To determine the bovine lactoferrin concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding bovine lactoferrin concentration. If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

1. The dose-response curve of this assay fits best to a sigmoidal 4-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4-parameter logistic function. It is important to make an appropriate mathematical adjustment to accommodate for the dilution factor.

2. Most microtiter plate readers perform automatic calculations of analyte concentration. The calibration curve is constructed by plotting the absorbance (Y) of calibrators versus log of the known concentration (X) of calibrators, using the 4-parameter function. Alternatively, the logit log function can be used to linearize the calibration curve (i.e. logit of absorbance (Y) is plotted versus log of the known concentration (X) of calibrators).

Measurement Range

The measurement range is 0.78 ng/mL to 50 ng/mL. Any sample reading higher than the highest standard should be diluted with Dilution Buffer in higher dilution and re-assayed. Dilution factors need to be taken into consideration in calculating the bovine lactoferrin concentration.

Troubleshooting

1. All samples and controls should be assayed in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.

2. Poor duplicates, accompanied by elevated values for wells containing no sample, indicate insufficient washing. If all instructions in the **Detailed Protocol** were followed accurately, such results indicate a need for washer maintenance.

3. Overall low signal may indicate that desiccation of the plate has occurred between the final wash and addition of Substrate Reagent. Do not allow the plate to dry out. Add Substrate Reagent immediately after wash.

Reagent Stability

All of the reagents included in the MBL Research Product **CircuLex Bovine Lactoferrin ELISA Kit have** been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, kit reagents should be stored at 4°C, except the reconstituted Lactoferrin Standard must be stored at below -70°C. Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

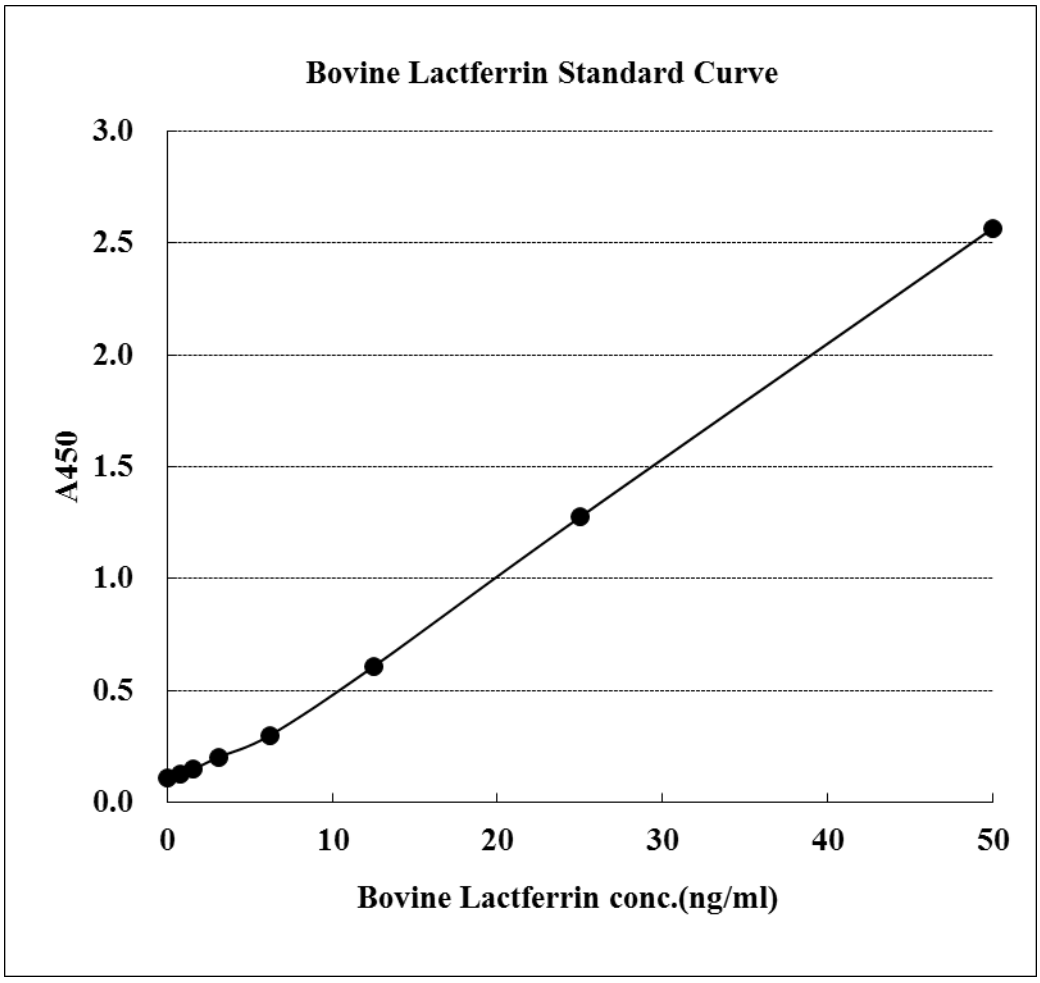
Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of bovine lactoferrin giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 0.78 ng/mL of sample.

* Dilution Buffer was pipetted into blank wells.

Typical Standard Curve



2. Precision

Intra-assay Precision (Precision within an assay)

Three samples* of known concentration were tested sixteen times on one plate to assess intra-assay precision.

• Intra-assay (Within-Run, n=16) CV=3.1-4.0 %

*Sample: Bovine milk

Bovine Lactferrin conc. (mg/mL)

Sample No.	Sample 1	Sample 2	Sample 3
1	2.96	1.92	0.23
2	2.81	1.92	0.22
3	2.86	1.92	0.22
4	2.89	1.98	0.22
5	2.88	1.95	0.22
6	2.96	2.03	0.23
7	2.89	1.94	0.23
8	3.20	1.99	0.23
9	2.94	1.90	0.24
10	2.84	1.82	0.24
11	2.72	1.84	0.23
12	2.80	1.88	0.24
13	2.87	1.87	0.24
14	2.95	1.98	0.23
15	2.88	2.02	0.24
16	3.10	1.96	0.24
MAX.	**3.20**	**2.03**	**0.24**
MIN.	**2.72**	**1.82**	**0.22**
MEAN	**2.91**	**1.93**	**0.23**
S.D.	**0.115**	**0.061**	**0.008**
C.V.	**4.0%**	**3.1%**	**3.3%**

Inter-assay Precision (Precision between assays)

Four samples* of known concentration were tested in five separate assays to assess inter-assay precision.

• Inter-assay (Run-to-Run, n=5) CV=3.2-6.0 %

*Sample: Bovine milk

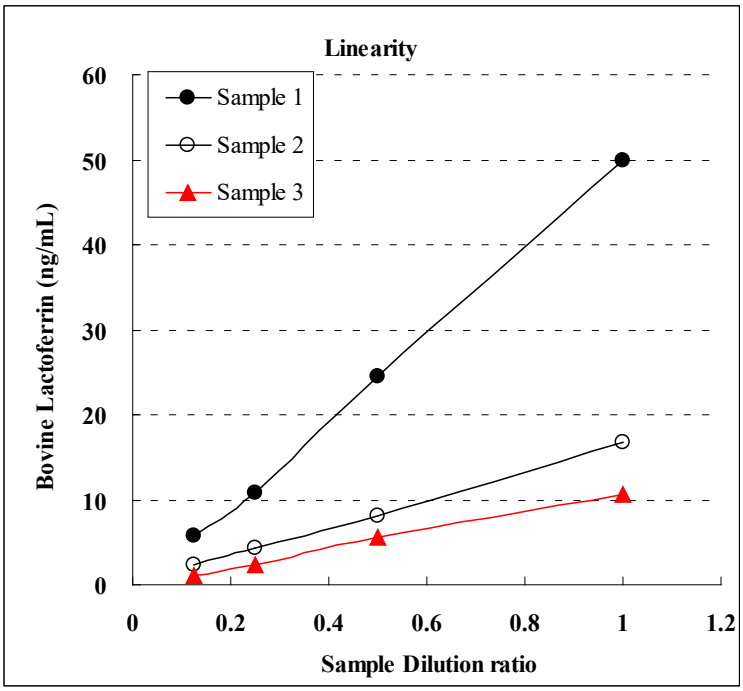
Bovine Lactferrin conc.(µg/ml)

	Sample 1	Sample 2	Sample 3	Sample 4
1	451.5	205.6	97.7	51.5
2	423.3	190.1	96.1	48.3
3	392.5	205.8	98.3	52.7
4	429.1	203.5	107.8	50.5
5	457.8	192.6	108.7	51.1
MAX.	**457.8**	**205.8**	**108.7**	**52.7**
MIN.	**392.5**	**190.1**	**96.1**	**48.3**
MEAN	**430.9**	**199.5**	**101.7**	**50.8**
S.D.	**25.9**	**7.6**	**6.0**	**1.6**
C.V.	**6.0%**	**3.8%**	**5.9%**	**3.2%**

3. Linearity

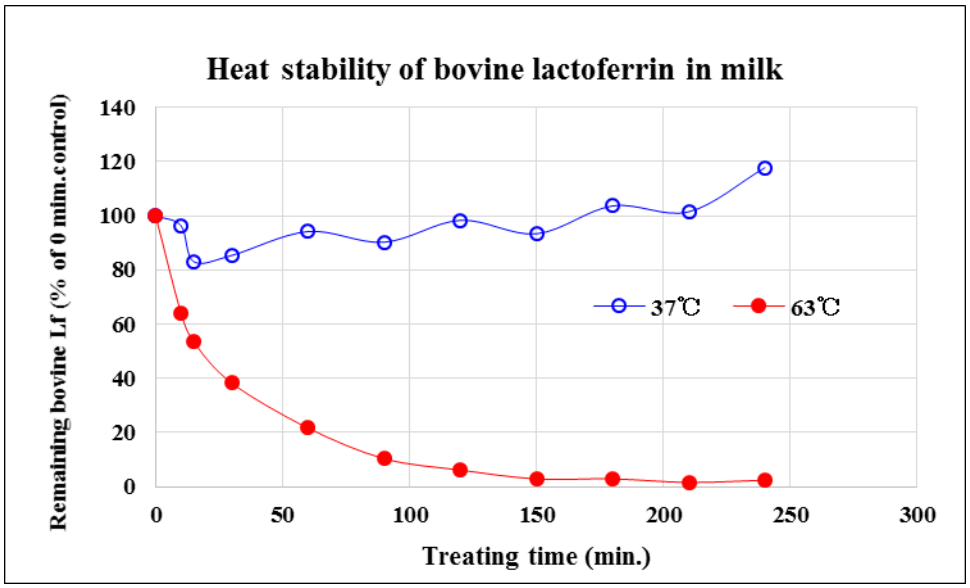
Three biological samples* were diluted with Dilution Buffer and assayed after dilution. The neat sample was set to 1.0. Please note all samples including the neat sample were 10,000-fold diluted as stated in the Assay Procedure. The results are summarized in the figure below.

*Sample: Bovine milk



Example of Test Results

Fig.1. Heat stability of bovine lactoferrin in milk measured by CircuLex Bovine Lactoferrin ELISA Kit



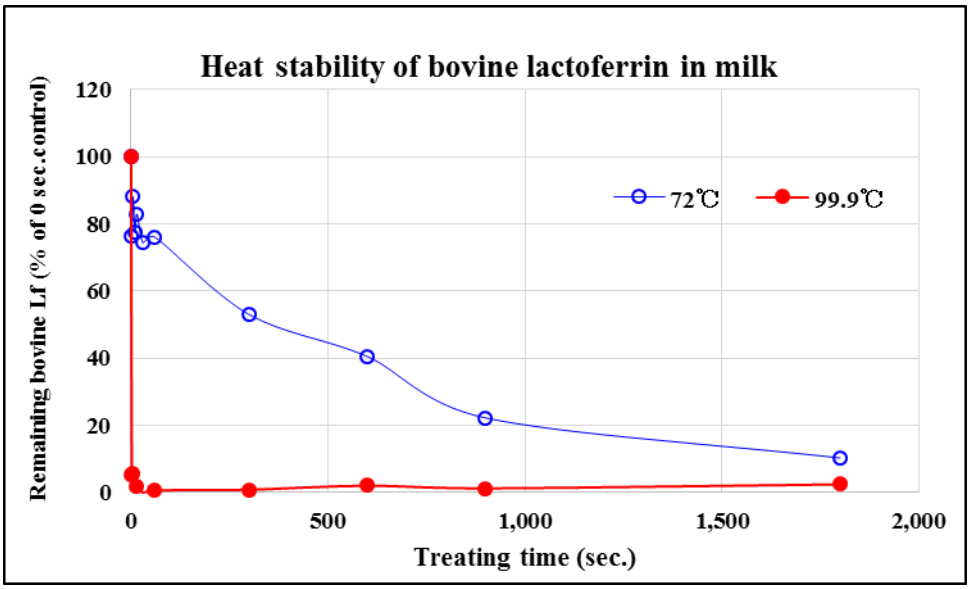
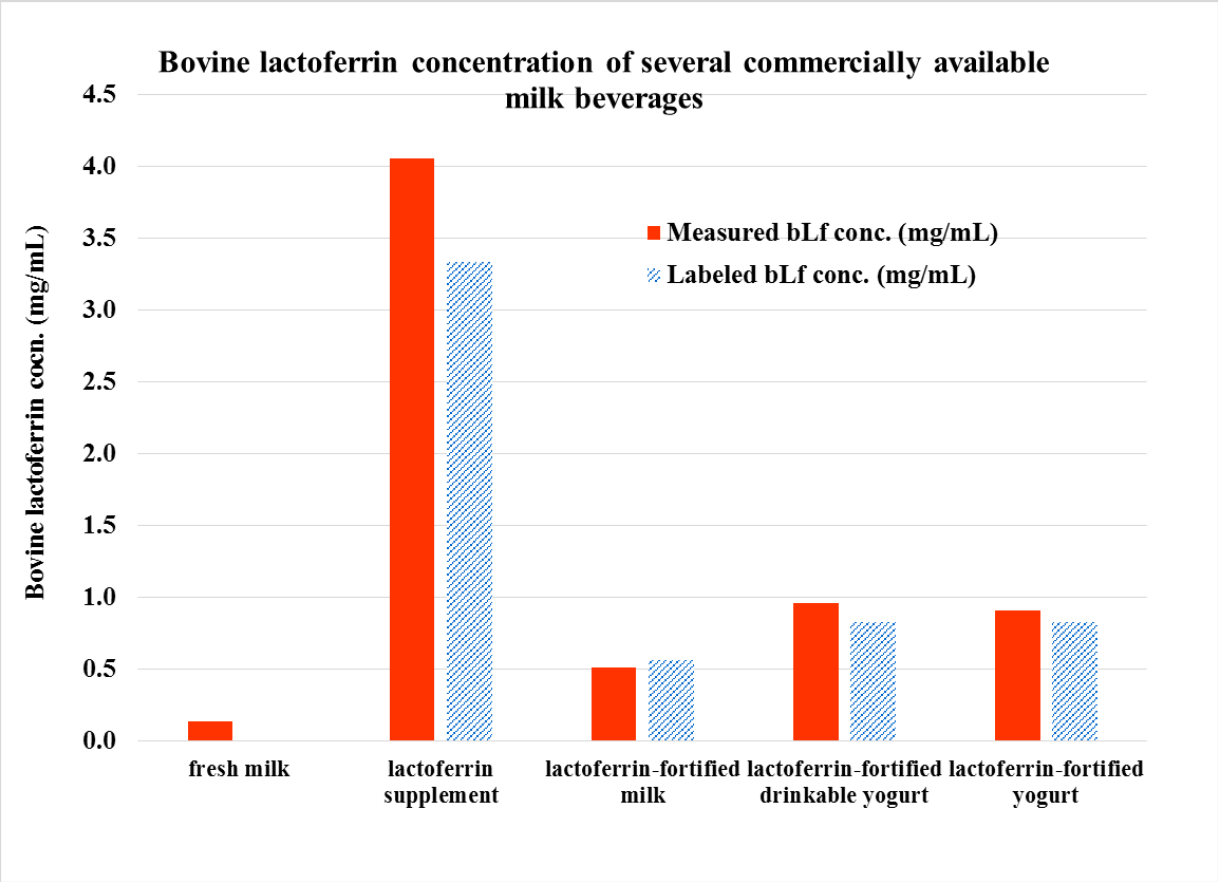


Fig.2. Bovine lactoferrin concentrations of several milk beverages measured by the CircuLex bovine Lactoferrin ELISA Kit



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