PERFORMANCE CHARACTERISTICS (continued)

Sample Recovery

High and low concentrations of purified bovine lactoferrin were spiked into each of 3 milk samples. Observed assay values compared to expected values ranged from 96 to 116%, indicating accurate quantification of lactoferrin in bovine milk.

Sample	Expected ng/ml	Observed ng/ml	Observed/ Expected
High Lactoferrin		58.0	
+ Milk A, 52.3 ng/ml	110.4	106.0	**96 %**
+ Milk B, 53.2ng/ml	111.2	116.0	**104 %**
+ NFD Milk, 64.2 ng/ml	109.8	127.0	**116 %**
Low Lactoferrin		16.6	
+ Milk A, 52.3 ng/ml	68.9	69.8	**101 %**
+ Milk B, 53.2ng/ml	69.8	75.4	**108 %**
+ NFD Milk, 64.2 ng/ml	76.2	85.0	**111 %**

ELISA Kit Components	Amount	Part No.
Anti-Bovine Lactoferrin Microwell Strip Plate	8-well strips (12)	8091
Bovine Lactoferrin Positive Control	0.65 ml	8092
Bovine Lactoferrin Standard 10 ng/ml	0.65 ml	8093B
Bovine Lactoferrin Standard 40 ng/ml	0.65 ml	8093C
Bovine Lactoferrin Standard 80 ng/ml	0.65 ml	8093D
Bovine Lactoferrin Standard 120 ng/ml	0.65 ml	8093E
Bovine Lactoferrin Standard 160 ng/ml	0.65 ml	8093F
Anti-Bovine Lactoferrin HRP Conjugate (100X)	0.15 ml	8094
Sample Diluent Concentrate (20X)	10 ml	SD-20T
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	M-8090

For more details please consult our web site (www.atzlabs.com) or contact us by email (customerservice@atzlabs.com)

Instruction Manual No. M-8090

Bovine Lactoferrin

ELISA Kit Cat. No. 8090

For Quantitative Determination of Bovine Lactoferrin in Milk and Fluids

ALPHA DIAGNOSTIC INTERNATIONAL

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INTENDED USE

The Bovine Lactoferrin ELISA Kit is an in vitro immunoassay for the quantification of lactoferrin in milk and colostrums, or in other appropriately qualified samples from tissue fluids (e.g., saliva, mucosa), or other solutions.

RESEARCH USE OF THE TEST

Lactoferrin is a natural, lactose-free, protein present in milk, which has a high affinity for iron. Lactoferrin is a versatile protein that can prevent the growth of pathogenic bacteria in the gut, and control cell or tissue damage. Lactoferrin also serves as a bio-regulator of iron and provides supportive functions for the immune system.

Iron is a key mineral required by many micro-organisms for maintenance and growth. Regulation of iron by lactoferrin in the digestive tract helps to maintain the correct balance of beneficial and harmful bacteria. Lactoferrin binds free iron and works with the immune system to achieve homeostasis. Lactoferrin delivers bound iron to beneficial bacteria and healthy cells by way of transferrin and helps maintain the current iron level by a complex biological process involving ferritin and transferrin. Lactoferrin decreases free, non-absorbed iron that would be otherwise available to pathogens.

Lactoferrin has also been shown to inhibit certain cytokine and interleukin production, which allows for the reduction of swelling and the increase of circulatory activity in the vicinity of injury.

PRINCIPLE OF THE TEST

The Bovine Lactoferrin ELISA kit is based on the binding of bovine lactoferrin in samples to two antibodies, one immobilized on the microtiter wells, and the other conjugated to horseradish peroxidase (HRP) enzyme. After a washing step, chromogenic substrate is added and color is developed by the enzymatic reaction of HRP on the TMB substrate, which is directly proportional to the amount of lactoferrin present in the sample. Stopping Solution is added to terminate the reaction, and absorbance at 450nm is then measured using an ELISA microtiter well reader. The concentration of lactoferrin in samples and control is calculated from a curve of standards containing known concentrations of lactoferrin.

STORAGE AND STABILITY

The microtiter well plate and all other reagents, if unopened, are stable at 2-8^{O}C until the expiration date printed on the box label. Stabilities of the working solutions are indicated under Reagent Preparation.

PERFORMANCE CHARACTERISTICS

Specificity
The antibodies used in this kit have been shown by immunoelectrophoresis and ELISA to react specifically with lactoferrin, and have essentially no reactivity with any other bovine serum or milk proteins. Human and goat milk showed essentially no reactivity at a 1:10 dilution.

Normal Range
Assay of lactoferrin in ten (10) bovine milk samples ranged from 27 to 165 ug/ml; 1% nonfat dry milk measured 13ug/ml. Each laboratory should determine expected values of its own testing population.

Precision
Samples containing low, medium and high concentrations of lactoferrin were assayed multiple times in the same assay (n=10) to provide within-assay precision, and as duplicates in multiple assays (n=5) to obtain between-assay reproducibility. Coefficient of variations were calculated for the concentrations using a point-to-point curve-fitting program.

Lactoferrin concentrations were measured with very good within-assay (3.4 to 5.3 %CV) and between-assay (3.4 to 5.3 %CV) reproducibility.

Sample	Lactoferrin ng/ml	Intra-assay %CV	Inter-assay %CV
Low Lactoferrin	13.9	**4.5**	**5.3**
Medium Lactoferrin	44.9	**3.8**	**4.2**
High Lactoferrin	95.0	**4.3**	**3.4**

Linearity of Dilution
Two (2) milk samples and one (1) nonfat dried milk sample were diluted to 2 levels for testing, and concordance of the assay values were compared. The mean recovery ranged from 93 to 96%, demonstrating linear dilution and equivalent quantification across the standard range.

Sample	Dilution	Assay Value ng/ml	Milk Value mg/ml	Concordance
Milk Sample A	1:500 1:2000	98.4 22.7	49.2 45.4	**96 %**
Milk Sample B	1:500 1:2000	47.3 13.6	23.7 27.2	**93 %**
Nonfat Dry Milk	1:150 1:1200	80.0 11.5	12.0 13.8	**93 %**

Continued on Page 7.

CALCULATION OF RESULTS

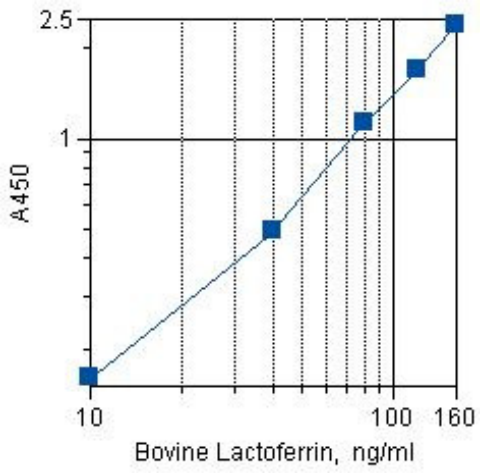
1. The results may be calculated using any immunoassay software package. The four-parameter curve-fit is recommended. If software is not available, lactoferrin concentrations may be determined as follows:
2. Calculate the mean OD of duplicate samples.
3. On graph paper plot the mean OD of the standards (y-axis) against the concentration (ng/ml) of lactoferrin (x-axis). Draw the best fit curve through these points to construct the standard curve. A point-to-point construction is most common and reliable.
4. The lactoferrin concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
5. Multiply the values obtained for the samples by the dilution factor of each sample.
6. Samples producing signals higher than the 160 ng/ml standard should be further diluted and re-assayed.

TYPICAL RESULTS

The following data are for illustration purposes only. A complete standard curve should be run in every assay to determine sample values.

Wells	Standards, Control & Samples	A450 nm	lactoferrin ng/ml
A1, A2	**Negative Diluent Control**	0.08	0
B1, B2	10 ng/ml **Standard**	0.16	10
C1, C2	40 ng/ml **Standard**	0.49	40
D1, D2	80 ng/ml **Standard**	1.12	80
E1, E2	120 ng/ml **Standard**	1.70	120
F1, F2	160 ng/ml **Standard**	2.40	160
G1, G2	**Positive Serum Control** [Value: 70 - 130 ng/ml]	1.36	101
H1, H2	**Sample** [Diluted 1:1000]	0.72	51.3
	Calculated: 1000-fold dilution x 51.3 ng/ml = **51.3** ug/ml in milk		

A typical assay Standard Curve (do not use for calculating sample values)



KIT CONTENTS

To Be Reconstituted: Store as indicated.

Component	Instructions for Use
Sample Diluent Concentrate (20x) Cat. No. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample Diluent** and store at 2-8°C until the kit lot expires or is used up.
Wash Solution Concentrate (100x) Cat. No. WB-100, 10ml	Dilute the entire volume 10ml + 990ml with distilled or deionized water into a clean stock bottle. Label as **Working Wash Solution** and store at RT until kit is used entirely.
Anti-Bovine lactoferrin - HRP Conjugate Concentrate (100x) Part No. 8094, 0.15ml	Peroxidase conjugated anti-bovine lactoferrin in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return concentrate to 2-8°C storage.

Ready For Use: Store as indicated on labels.

Component	Part No.	Amt	Contents
Anti-Bovine Lactoferrin Microwell Strip Plate	8091	8-well strips (12)	Coated with purified anti-bovine lactoferrin antibodies.
Bovine Lactoferrin Standards			
10 ng/ml 40 ng/ml 80 ng/ml 120 ng/ml 160 ng/ml	8093B 8093C 8093D 8093E 8093F	0.65 ml 0.65 ml 0.65 ml 0.65 ml 0.65 ml	Five (5) vials, each containing calibrated lactoferrin concentrations; diluted in buffer with protein, detergents and antimicrobial as stabilizers.
Positive Control [lactoferrin] range on label	8092	0.65 ml	Bovine lactoferrin with stated concentration range; diluted in buffer with protein, detergents and antimicrobial as stabilizers.
TMB Substrate	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80101	12 ml	1% sulfuric acid.

Materials Required But Not Provided:

- Pipettors and pipettes that deliver 100ul and 1-10ml. A multi-channel pipettor is recommended.
- Disposable glass or plastic 5-15ml tubes for diluting samples and anti-bovine lactoferrin-HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate and Sample Diluent concentrate; 200ml to 1L.
- Stock bottle to store diluted Wash Solution; 200ml to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength.

SPECIMEN COLLECTION AND HANDLING

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 Working Sample Diluent. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage. Avoid freeze-thaw cycles.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Standards, Controls, Sample Diluent, and Anti-bovine lactoferrin-HRP contain Bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains 1% sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water.
MSDS for TMB, sulfuric acid and BND, if not already on file, can be requested or obtained from the ADI website.

QUALITY CONTROL

Reagents Accurate and reproducible assay results rely on proper storage, handling and control of reagent and sample temperature. Store all reagents as indicated, and warm to room temperature only those to be used in the assay. Shelf-life of the critical reagents and samples will diminish with extended exposure to non-refrigeration, resulting in inaccurate assay results. All solutions should be clear. Cloudiness or particulates are indications of reagent contamination or instability and may interfere with proper performance of the assay. Do not use.

Sample Controls A Positive Serum Control is provided with the kit, assigned with a lactoferrin concentration value range. Recovery in this range is an indicator of proper assay performance. Each lab should also assay internal control samples, which represent the lab's expected sample population and that are maintained stabilized. A Negative Diluent Control should also be run; blank signal should be <0.3 OD.

Standard Curve The signal generated by the standards should be continuously increasing in OD from the lowest Standard to the highest Standard, with a difference greater than 1.2 OD. Non-continuously increasing or low signals may indicate problems with technique, protocol directions and/or reagent preparation, use or stability. A Negative Diluent Control should be of lower signal than the lowest standard. Do not rely on results generated from an assay with these issues.

Technique Accurate and reproducible assay results rely on good lab technique regarding pipetting, plate washing and handling of samples and reagents.

Equipment Precision of results relies on uniform and effective washing techniques; an automatic washer is recommended. ELISA reader and pipettes should be properly calibrated.

ASSAY PROCEDURE

Bring all reagents to room temperature (18-30^{o} C) equilibration (at least 30 minutes).

DILUTE Samples in Working Sample Diluent. Dilutions of about 500-fold are appropriate for most normal milk samples. For accuracy, two dilution steps are recommended, as follows:
1) 20ul serum + 380ul diluent = [1:20],
2) 20ul [1:20] + 480ul diluent = **[1:500]**.

DO NOT diluted the Standards or Control.

ALL STEPS ARE PERFORMED AT ROOM TEMPERATURE. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

1. Set-up

- Determine the number of wells for the assay run. Duplicates are recommended, including 10 Standard wells and 2 wells for each sample and control to be assayed.
- Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
- Add 200-300ul Working Wash Solution to each well and let stand for 5 to 30 minutes before sample addition.
- Aspirate the liquid and pat dry on a paper towel.

2. 1st Incubation [100ul – 60 min; 4 washes]

- Add 100ul of standards, samples and controls each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for 60 minutes.
- Wash wells 4 times and pat dry on fresh paper towels. As an alternative, an automatic plate washer is recommended. Improper washes may lead to falsely elevated signals and poor reproducibility.

3. 2nd Incubation [100ul – 30 min; 5 washes]

- Add 100ul of diluted Anti-bovine lactoferrin-HRP Conjugate to each well.
- Incubate for 30 minutes.
- Wash wells 5 times as in step 2.

4. Substrate Incubation [100ul – 15 min]

- Add 100ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
- Incubate for 15 minutes in the dark, e.g., place in a drawer or closet.
  Note: If your microplate reader does not register optical density (OD) above 2.0, incubate for less time, assuring the top standard does not surpass 2 OD.

5. Stop Step [Stop: 100ul]

- Add 100ul of Stop Solution to each well.
- Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

6. Absorbance Reading

- Use any commercially available microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
- Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.