

# Bovine Lactoferrin ELISA Quantitation Set

Cat. No. E10-126

## **Components Supplied**

- Affinity purified Bovine Lactoferrin Coating Antibody (A10-126A), 1 ml
- Bovine Lactoferrin Calibrator (RC10-126), 0.1 ml
- HRP Conjugated Bovine Lactoferrin Detection Antibody (A10-126P), 0.1 ml

## **Introduction**

Enzyme linked immunosorbent assay (ELISA) for the detection of Bovine Lactoferrin in milk, colostrum, or plasma. Other biological fluids that contain bovine Lactoferrin, such as urine, tears, or saliva, may be suitable samples. The Set contains sufficient components to perform 1000 single well assays.

The set performance has been optimized for the stated protocol using the materials listed and standard dilutions from 500 – 7.8 ng/ml of Bovine Lactoferrin. *For alternative assay conditions, the operator must determine appropriate dilutions of reagents.*

Country of Origin: USA

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## Procedure Overview

1. Add 100 µl of diluted coating antibody to each well.  
Note: Run each standard or sample in duplicate.
- ↓
2. Incubate at room temperature (20-25°C) for 1 hour.
- ↓
3. Wash plate FIVE times.
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4. Add 200 µl of Blocking Solution to each well.
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5. Incubate at room temperature for 30 minutes.
- ↓
6. Wash plate FIVE times.
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7. Add 100 µl of standard or sample to well.
- ↓
8. Incubate at room temperature for 1 hour.
- ↓
9. Wash plate FIVE times.
- ↓
10. Add 100 µl of diluted HRP detection antibody to each well.
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11. Incubate at room temperature for 1 hour.
- ↓
12. Wash plate FIVE times.
- ↓
13. Add 100 µl of TMB Substrate Solution to each well.
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14. Develop the plate in the dark at room temperature for 15 minutes.
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15. Stop reaction by adding 100 µl of Stop Solution to each well.
- ↓
16. Measure absorbance on a plate reader at 450 nm.

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### **Plates, Buffers and Substrate not provided**

- 96-well plate (Cat. no. E105)
- ELISA Coating Buffer (Cat. No. E107)
- ELISA Wash Solution (Cat. No. E106)
- Blocking Buffer, same as the ELISA Wash Solution
- Sample/Conjugate Diluent, same as the ELISA Wash Solution
- Enzyme Substrate, TMB (Cat. No. E102)
- ELISA Stop Solution (Cat. No. E115)

The above products may be bought individually or bought together in the ELISA Starter Accessory Kit II (Cat. No. E103). ELISA Stop Solution (Cat. No. E115) is not included in the kit and is sold separately. The E103 contains 10 x 96-well plates.

Buffers may be prepared in your lab according to the formulations specified under Buffer Preparation of this protocol.

### **Additional Materials Required**

- Ultrapure water
- Precision pipettors, with disposable plastic tips
- Polypropylene, polyethylene or glass tubes to prepare standard and samples
- Containers to prepare buffers
- An aspiration device or an automated 96-well plate washer
- Disposable reagent reservoirs
- A standard microtiter plate reader for measuring absorbance at 450 nm

### **Precautions**

- Store all reagents at 2-8°C. Do not freeze reagents.
- All reagents must be at room temperature (20-25°C) before use.
- Vigorous plate washing is essential.
- Use new disposable pipette tips for each transfer to avoid cross-contamination.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can easily become contaminated thereby causing assay variability.
- Take care not to contaminate the TMB Solution. Do not expose TMB Substrate solution to glass, foil, or metal. If the solution is blue before use, DO NOT USE IT.

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## **Buffer Preparation**

Prepare the following buffers from the ELISA Starter Accessory Kit (E103) or your lab:

- Coating Buffer, 0.05 M Carbonate-Bicarbonate, pH 9.6
- ELISA Wash Solution, 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0
- ELISA Blocking Solution, 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0
- Sample/Conjugate Diluent, 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0
- Enzyme Substrate, TMB
- ELISA Stop Solution, 0.18 M H<sub>2</sub>SO<sub>4</sub>

**Bovine Serum Albumin should not be used as the blocking agent. TBS with 0.05% Tween 20 is adequate.**

## **Sample, Reagent, and Standard Handling and Preparation**

### **Sample Handling**

- Milk may be tested in this ELISA. Other fluids containing bovine Lactoferrin may be tested but interpretation is subject to researcher.
- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions when handling and disposing of infectious agents.
- 100 µl of sample or standard is required per well.
- Samples must be assayed in duplicate each time the assay is performed.
- Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles when storing samples.
- If particulate is present in samples, centrifuge prior to analysis.
- If samples are clotted, grossly hemolyzed, lipemic, or the integrity of the sample is of concern, make a note on the Plate Template and interpret results with caution.

### **Sample Dilution**

- Dilute the samples, based on the expected concentration of the analyte, to fall within the concentration range of the standards.

### **Standard Dilution**

- Standard should be treated as a biological material and universal precautions should be followed. Follow the recommended dilutions in the table provided under Standards and Samples.

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## **Plate Washing**

1. Fill each well with ELISA Wash Solution
2. Remove ELISA Wash Solution by aspiration. Aspirate plate contents.
3. Repeat procedure four additional times for a total of FIVE washes. Blot plate onto paper towels or other absorbent material.

**Note:** For automated washing, aspirate plate contents from all wells and fill wells with Wash Buffer. Repeat procedure four additional times for a total of FIVE washes. Blot plate onto paper towels or other absorbent material.

Take care to avoid microbial contamination of equipment. Automated plate washers can easily become contaminated thereby causing assay variability.

## **Plate Coating and Blocking**

Determine the number of wells required. Standards, samples, blanks and/or controls should be analyzed in duplicate.

1. Dilute 1 mcl affinity purified antibody (A10-126A) to 100 mcl Coating Buffer for each well to be coated. (Example: for 32 wells dilute 34 mcl to 3.4 ml)
2. Add 100 mcl of diluted antibody to each well.
3. Incubate coated wells at room temperature (20-25°C) for 60 minutes.
4. After incubation, aspirate the antibody solution from each well.
5. Wash FIVE times as described in the Plate Washing section.
6. Add 200 mcl of Blocking Solution to each well.
7. Incubate 30 minutes at room temperature (20-25°C).
8. After incubation, remove the Blocking Solution and wash each well FIVE times as described in the Plate Washing section.
9. Proceed with remainder of assay.

Note: Plates can be held for up to 24 hours after the plate coating and blocking steps. Leave Blocking Solution in wells after step 6, cover and place at 2-8°C. To continue assay, remove Blocking Solution, wash and proceed with remainder of assay.

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## **Standards and Samples**

Dilute the standards in Sample/Conjugate Diluent according to the chart below:

<b>Standard</b>	<b>ng/ml</b>	<b>RC10-126-7 (1 mg/ml Lactoferrin)</b>	<b>Sample Diluent</b>
1	500	5 mcl	10 ml
2	250	500 mcl from std 1	500 mcl
3	125	500 mcl from std 2	500 mcl
4	62.5	500 mcl from std 3	500 mcl
5	31.25	500 mcl from std 4	500 mcl
6	15.625	500 mcl from std 5	500 mcl
7	7.8	500 mcl from std 6	500 mcl
8	0	Blank	500 mcl

1. Label eight (8) tubes, one for each standard curve point: 500 ng/ml, 250 ng/ml, 125 ng/ml, 62.5 ng/ml, 31.25 ng/ml, 15.625 ng/ml, 7.8 ng/ml, and 0 ng/ml (blank).
2. Prepare initial dilution of 500 ng/ml by diluting 5 mcl of Bovine Lactoferrin Calibrator (RC10-126-7) with 10 ml of Sample/Conjugate Diluent. Mix well.
3. Pipette 500 mcl in remaining tubes.
4. Serial dilute the 500 ng/ml standard 1:1 with Sample/Conjugate Diluent. Perform dilution by mixing 500 mcl of the previous standard with 500 mcl of Sample/Conjugate Diluent. Continue until standard value of 7.8 ng/ml is reached.
5. Use Sample/Conjugate Diluent only as the zero standard value.
6. Dilute the samples, based on the expected concentration of the analyte, to fall within the concentration range of the standards.
7. Transfer 100 mcl of standard or sample to assigned wells.
8. Incubate plate 60 minutes at room temperature (20-25°C).
9. After incubation, remove samples and standards and wash FIVE times as described in the Plate Washing section.

## **HRP Detection Antibody**

1. Dilute the HRP Detection Antibody (A10-126P) in Sample/Conjugate Diluent. Recommended starting dilution is 1:100,000. If OD value for high standard is below expected 1.8–2.2, the HRP Detection antibody can be diluted less to increase the OD value.
2. Transfer 100 mcl to each well.
3. Incubate 60 minutes at room temperature (20-25°C).
4. After incubation, remove HRP Detection Antibody and wash FIVE times as described in the Plate Washing section.

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## **TMB Substrate Incubation and Reaction Stop**

1. Prepare the substrate solution according to the manufacturer's recommendation. TMB substrate in the ELISA Starter Accessory Kit is supplied as a ready to use solution. Only remove the required amount of TMB Substrate Solution for the number of wells being used.
2. Do NOT use a glass pipette to measure the TMB Substrate Solution. Do NOT cover the plate with aluminum foil or metalized mylar. Do NOT return leftover TMB Substrate to bottle. Do NOT contaminate the unused TMB Substrate Solution. If the solution is blue before use, DO NOT USE IT!
3. Add 100  $\mu$ l of TMB Substrate Solution into each well.
4. Allow the enzymatic color reaction to develop at room temperature (20-25°C) in the dark for 15 minutes. The substrate reaction yields a blue solution.
5. After 15 minutes, stop the reaction by adding 100  $\mu$ l of ELISA Stop Solution (0.18 M H<sub>2</sub>SO<sub>4</sub>). Tap plate gently to mix. The solution in the wells should change from blue to yellow.

## **Absorbance Measurement**

**Note:** Evaluate the plate within 30 minutes of stopping the reaction.

1. Wipe underside of wells with a lint-free tissue.
2. Measure the absorbance on an ELISA plate reader set at 450 nm.

## **Calculation of Results**

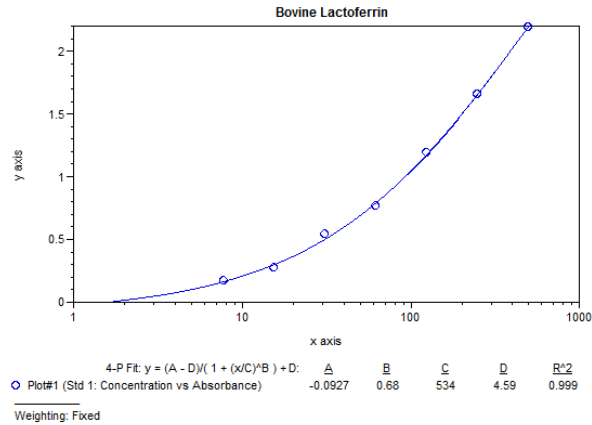
1. Duplicate absorbance values should be within 10% of each other. Care should be taken when interpreting data with differences in absorbance values greater than 10%.
2. Prepare a standard curve to determine the amount of Bovine Lactoferrin in an unknown sample. Plot the average absorbance value minus the blank value for each standard concentration on the vertical (Y) axis versus the corresponding Bovine Lactoferrin concentration on the horizontal (X) axis using graph paper or curve-fitting software.
3. Calculate the Bovine Lactoferrin concentration in unknown samples using the prepared standard curve. Determine the amount of Bovine Lactoferrin in each unknown sample by noting the Bovine Lactoferrin concentration (X axis) that correlates with the absorbance value (Y axis) obtained for the unknown sample.
4. If the sample was diluted, multiply the Bovine Lactoferrin concentration obtained by the dilution factor to determine the amount of Bovine Lactoferrin in the undiluted sample.

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## **Performance Characteristics**

### **Typical Standard Curve**

This typical standard curve was generated using Bovine Lactoferrin ELISA Quantitation Set Protocol. This standard curve is for demonstration only. A standard curve must be generated for each assay. Curve was generated as a 4-parameter curve fit using Soft-Max Pro.



**Assay Range:** 7.8 – 500 ng/ml

Suggested standard curve points are 500 ng/ml, 250 ng/ml, 125 ng/ml, 62.5 ng/ml, 31.25 ng/ml, 15.625 ng/ml, 7.8 ng/ml and 0 ng/ml (blank).

### **Specificity**

By immunoelectrophoresis and ELISA the antibodies in this set react specifically with Bovine Lactoferrin, not with Bovine immunoglobulins or other Bovine milk proteins. Cross-reactivity with other species has not been tested.

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## **Troubleshooting**

### **Problem: Low absorbance**

- Incorrect dilutions or pipetting errors
- Improper incubation times
- Wrong filter on microtiter reader. Wavelength should be 450 nm for TMB.
- Set materials or reagents are contaminated or expired.
- Incorrect reagents used.
- Dilute the HRP Detection Antibody less.

### **Problem: High Absorbance**

- Cross contamination from other samples or positive control
- Incorrect dilutions or pipetting errors
- Improper washing
- Wrong filter on microtiter reader. Wavelength should be 450 nm for TMB.
- Contaminated buffers or enzyme substrate
- Improper incubation times
- Set materials or reagents are contaminated or expired.
- Dilute the HRP Detection Antibody more.

### **Problem: Poor Duplicates**

- Poor mixing of specimens
- Incorrect dilutions or pipetting errors
- Technical error
- Inconsistency in following ELISA protocol
- Inefficient washing

### **Problem: All wells are positive**

- Contaminated buffers or enzyme substrate
- Incorrect dilutions or pipetting errors
- Set materials or reagents are contaminated or expired.
- Inefficient washing

### **Problem: All wells are negative**

- Procedure not followed correctly
- Contaminated buffers or enzyme substrate
- Contaminated Conjugate
- Set materials or reagents are contaminated or expired.

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## **Technical Hints**

- When preparing coating buffer from the gel capsule, break the capsule apart and pour ingredients into water. Do not place gel capsule into water. The gelatin from the capsule interferes with the binding of the coating antibody to the plate.
- Capture antibody diluted with coating buffer should be added to wells immediately.
- Coated (covered) plates are stable overnight at 4°C.
- Check all buffers for contamination and expiration. When trouble shooting, it may be helpful to start with all new buffers. Make buffers in new or properly cleaned vessels.
- Sodium Azide should not be added to any of the buffers.
- Dilutions should be made shortly before application and immediately applied to appropriate wells. Do not save extra diluted standards or samples for future assays.
- Wash buffer should be aspirated from wells. Pouring/Dumping wash buffer from wells may lead to cross contamination.
- Excess antibody/analyte should be wiped from pipettes tips when making dilutions.
- Incubation time of the TMB Substrate will depend on the intensity of the color change. The high standard should have an O.D. reading of 1.8 – 2.2 and the O.D. reading of the low standard should be above background. Stop solution should be added to the plate in the same order as the TMB Substrate.

## **Warranty**

Products are warranted by Bethyl Laboratories, Inc. to meet stated product specifications and to conform to label descriptions when used, handled and stored according to instructions. Unless otherwise stated, this warranty is limited to one year from date of sale. Bethyl Laboratories sole liability for the product is limited to replacement of the product or refund of the purchase price. Bethyl Laboratories products are supplied for research applications. They are not intended for medicinal, diagnostic or therapeutic use. The products may not be resold, modified for resale or used to manufacture commercial products without prior written approval from Bethyl Laboratories, Inc.

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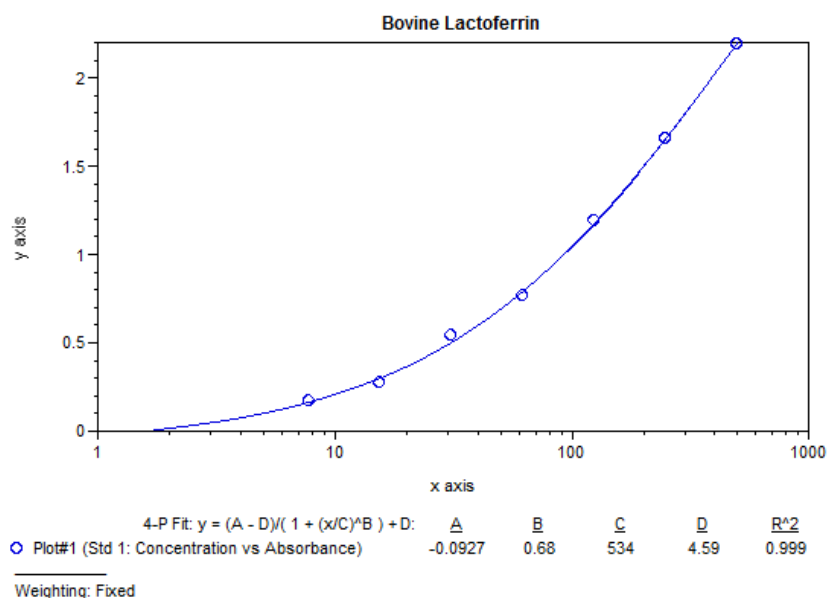
## Plate Templates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

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## Bovine Lactoferrin ELISA Quantitation Set



Concentration (ng/ml)	Abs 450 nm	Calculated Concentration
500	2.262	501.62
	2.128	
250	1.662	249.43
	1.648	
125	1.183	127.57
	1.197	
62.5	0.749	58.47
	0.766	
31.25	0.532	34.82
	0.546	
15.6	0.296	14.15
	0.247	
7.8	0.177	8.08
	0.148	

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