



KAMIYA BIOMEDICAL COMPANY

Human Lactoferrin ELISA

**For the quantitative determination of lactoferrin
in human biological samples**

Cat. No. KT-489

For Research Use Only. Not for use in diagnostic procedures.

PRODUCT INFORMATION**Human Lactoferrin ELISA**
Cat. No. KT-489**INTENDED USE**

The Human Lactoferrin ELISA is a highly sensitive two-site enzyme-linked immunoassay (ELISA) for the quantitative determination of lactoferrin in human biological samples. For research use only. Not for use in diagnostic procedures.

INTRODUCTION

Lactoferrin (LF) is a multifunctional iron-binding protein with antimicrobial activities. It is found in milk and other mucosal secretions. Lactoferrin is known to be part of the innate defense against infection and inflammation and also has function in regulation of iron absorption.

PRINCIPLE

The principle of the double antibody sandwich ELISA is represented in Figure 1. In this assay the lactoferrin present in samples reacts with the anti-lactoferrin antibodies which have been adsorbed to the surface of polystyrene microtiter wells. After the removal of unbound proteins by washing, anti-lactoferrin antibodies conjugated with horseradish peroxidase (HRP), are added. These enzyme-labeled antibodies form complexes with the previously bound lactoferrin. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of lactoferrin in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of lactoferrin in the test sample. The quantity of lactoferrin in the test sample can be interpolated from the calibration curve constructed from the calibrators, and corrected for sample dilution.

Figure 1.**COMPONENTS**

1. Diluent Concentrate
One bottle containing 50 mL of a 5X concentrated diluent running buffer.
2. Wash Solution Concentrate
One bottle containing 50 mL of a 20X concentrated wash solution.

3. Enzyme-Antibody Conjugate Concentrate
One vial containing 150 μ L of a 100X concentrated affinity-purified anti-human lactoferrin antibody conjugated with HRP in stabilizing buffer.
4. TMB Substrate Solution
One bottle containing 12 mL of TMB and hydrogen peroxide in citric acid buffer at pH 3.3.
5. Stop Solution
One bottle containing 12 mL of 0.3 M sulfuric acid.
WARNING: Avoid contact with skin.
6. Microtiter Plate
Twelve removable eight-well strips in well holder frame. Wells are coated with affinity-purified anti-human lactoferrin.
7. Human Lactoferrin Calibrator
One vial containing a lyophilized Human Lactoferrin Calibrator.

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes (2 μ L to 200 μ L) for making and dispensing dilutions
- Test tubes
- Microplate washer/aspirator
- Distilled or de-ionized H₂O
- Microplate reader
- Assorted glassware for the preparation of reagents and buffer solutions
- Timer
- Centrifuge for sample collection
- Anticoagulant for plasma collection

PRECAUTIONS

1. Read the instructions carefully before beginning the assay.
2. This kit is for research use only.
3. Great care has been taken to ensure the quality and reliability of this product. However, it is possible that in certain cases, unusual results may be obtained due to high levels of interfering factors.
4. No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.
5. Azide and thimerosal at concentrations higher than 0.1% inhibit the enzyme reaction.
6. Other precautions:
 - Do not interchange kit components from different lots.
 - Do not use kit components beyond the expiration date.
 - Protect reagents from direct sunlight.
 - Do not pipette by mouth.
 - Do not eat, drink, smoke or apply cosmetics where reagents are used.
 - Avoid all contact with the reagents by using gloves.
 - Stop solution contains diluted sulfuric acid. Irritation to eyes and skin is possible. Flush with water after contact.

REAGENT PREPARATION

1. Diluent Concentrate
The Diluent solution supplied is a 5X concentrate and must be diluted 1:5 with distilled or de-ionized water.
2. Wash Solution Concentrate
The Wash Solution supplied is a 20X concentrate and must be diluted 1:20 with distilled or de-ionized water. Crystal formation in the concentrate is not uncommon when storage temperatures are low. Warming of the concentrate to 30-35°C before dilution can dissolve crystals.
3. Enzyme-Antibody Conjugate Concentrate
Calculate the required amount of working conjugate solution for each microtiter plate test strip by adding 10 μ L Enzyme-Antibody Conjugate to 990 μ L of 1X Diluent for each test strip to be used for testing. Dilute immediately before use and protect from light. Mix uniformly, but gently. Avoid foaming.

4. TMB Substrate Solution
Ready to use as supplied.
5. Stop Solution
Ready to use as supplied.
6. Microtiter Plate
Ready to use as supplied. Unseal microtiter pouch and remove plate from pouch. Remove all strips and wells that will not be used in the assay and place back in pouch and re-seal along with desiccant.
7. Human Lactoferrin Calibrator
Add 1.0 mL of distilled or de-ionized water to the lyophilized Human Lactoferrin Calibrator and mix gently until dissolved. The calibrator is now at a concentration of 18.40 µg/mL (**the reconstituted calibrator should be aliquoted and frozen immediately if future use is intended**). Human Lactoferrin Calibrators need to be prepared immediately prior to use (see chart below). Mix well between each step. Avoid foaming.

Calibrator	Concentration (ng/mL)	Calibrator Volume added to 1X Diluent	→ Volume of 1X Diluent
6	100	5 µL Human Lactoferrin Calibrator	915 µL
5	50	300 µL Calibrator 6	300 µL
4	25	300 µL Calibrator 5	300 µL
3	12.5	300 µL Calibrator 4	300 µL
2	6.25	300 µL Calibrator 3	300 µL
1	3.13	300 µL Calibrator 2	300 µL
0	0		600 µL

STORAGE AND STABILITY

1. Complete Kit
The expiration date for the kit is stated on the outer label. The recommended storage temperature is 4°C. **Note: See long term storage recommendations below for the Human Lactoferrin Calibrator.**
2. Diluent
The 5X Diluent Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions should be stored at 4°C.
3. Wash Solution
The 20X Wash Solution Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions should be stored at 4°C.
4. Enzyme-Antibody Conjugate
Undiluted anti-lactoferrin-HRP conjugate should be stored at 4°C in the dark and **diluted immediately prior to use**. The undiluted conjugate is stable until the expiration date.
5. TMB Substrate Solution
The TMB Substrate Solution should be stored at 4°C in the dark and is stable until the expiration date. Protect from light.
6. Stop Solution
The Stop Solution should be stored at 4°C and is stable until the expiration date.
7. Microtiter Plate
Anti-human lactoferrin coated wells are stable until the expiration date, and should be stored at 4°C in the sealed foil pouch with desiccant pack.
8. Human Lactoferrin Calibrator
The lyophilized Human Lactoferrin Calibrator should be stored at 4°C or frozen until reconstituted. The reconstituted calibrator should be aliquoted out and stored frozen (avoid multiple freeze-thaw cycles). The working calibrator solutions should be prepared immediately prior to use.

INDICATIONS OF INSTABILITY

If the test is performing correctly, the results observed with the calibrator solutions should be within 20% of the expected values.

SPECIMEN COLLECTION AND HANDLING

Blood should be collected by venipuncture and the serum separated from the cells, after clot formation, by centrifugation. For plasma samples, blood should be collected into a container with an anticoagulant and then centrifuged. Care should be taken to minimize hemolysis, excessive hemolysis can impact your results. Assay immediately or aliquot and store samples at -20°C. Avoid repeated freezing/thawing.

For any sample that might contain pathogens, care must be taken to prevent contact with open wounds. No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.

ASSAY PROTOCOL

Dilution of Samples

Due to the high sensitive nature of the assay each sample should be diluted before use for a normal assay. A 1:20 dilution of serum or plasma is appropriate for most samples while human milk samples may need to be diluted 1:50,000. For absolute quantification of samples that yield results outside the range of the calibration 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.**

To prepare a 1:20 dilution of sample, transfer 20 μL of sample to 380 μL of 1X Diluent. This gives you a 1:20 dilution. Mix thoroughly. To prepare a 1:50,000 dilution of sample, transfer 5 μL of sample to 495 μL of 1X Diluent. This gives you a 1:100 dilution. Mix thoroughly. Next, dilute the 1:100 samples by transferring 2 μL to 998 μL of 1X Diluent. You now have a 1:50,000 dilution of your sample. Mix thoroughly at each stage.

Procedure

Bring all reagents to RT before use.

1. Pipette 100 μL of
 - Calibrator 0 (0.0 ng/mL) in duplicate
 - Calibrator 1 (3.13 ng/mL) in duplicate
 - Calibrator 2 (6.25 ng/mL) in duplicate
 - Calibrator 3 (12.5 ng/mL) in duplicate
 - Calibrator 4 (25 ng/mL) in duplicate
 - Calibrator 5 (50 ng/mL) in duplicate
 - Calibrator 6 (100 ng/mL) in duplicate
2. Pipette 100 μL of diluted sample (in duplicate) into pre-designated wells.
3. Incubate the Microtiter Plate at 22°C (RT) for thirty (30 \pm 2) minutes. Keep plate covered and level during incubation.
4. Following incubation, aspirate the contents of the wells.
5. Completely fill each well with appropriately diluted Wash Solution and aspirate. Repeat three times, for a total of four washes. If washing manually: completely fill wells with diluted Wash Solution, invert the plate and pour/shake out the contents in a waste container. Follow this by sharply striking the wells on absorbent paper to remove residual Wash Solution. Repeat three times for a total of four washes.
6. Pipette 100 μL of appropriately diluted Enzyme-Antibody Conjugate to each well. Incubate at 22°C (RT) for thirty (30 \pm 2) minutes. Keep plate covered in the dark and level during incubation.
7. Wash and blot the wells as described in Steps 4 and 5.
8. Pipette 100 μL of TMB Substrate Solution into each well.

9. Incubate in the dark at RT for precisely ten (10) minutes.
10. After ten minutes, add 100 μ L of Stop Solution to each well.
11. Determine the absorbance at 450 nm of the contents of each well within 30 minutes. Calibrate the plate reader to manufacturer's specifications.

The absorbance of the final reaction mixture can be measured up to 2 hours after the addition of the Stop Solution. However, good laboratory practice dictates that the measurement be made as soon as possible.

RESULTS

1. Subtract the average background value from the test values for each sample.
2. Using the results observed for the calibrators construct a calibration curve. The appropriate curve fit is that of a four-parameter logistics curve, although a second order polynomial (quadratic) or other curve fits may also be used.
3. Interpolate test sample values from calibration curve. Correct for sample dilution factor to arrive at lactoferrin concentration in original sample.

PERFORMANCE CHARACTERISTICS

In accord with good laboratory practice, the assays for specific lactoferrin require meticulous quality control. Each laboratory should use routine quality control procedures to establish inter- and intra-assay precision and performance characteristics.

LIMITATION OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the information contained in the package insert instructions and with adherence to good laboratory practice.
2. Factors that might affect the performance of the assay include proper instrument function, cleanliness of glassware, quality of distilled or de-ionized water, and accuracy of reagent and sample pipettings, washing technique, incubation time or temperature.

FOR RESEARCH USE ONLY

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