Human Lactoferrin ELISA Kit

Introduction

Lactoferrin is an 80 kDa iron-binding glycoprotein produced by many exocrine glands with a major constituent in the secondary granules of neutroplilic leukocytes. Serum lactoferrin concentration is much higher during inflammation (1). Lactoferrin is known to be an immune modulator or enhancer due to specific receptors for lactoferrin that are found on many key immune cells such as lymphocytes, monocytes and macrophages, and is known to be directly involved in the up-regulation of natural killer (NK) cell activity (2). Lactoferrin is present in maternal milk, saliva, tears, vaginal secretions, semen, bronchoalveolar lavage fluid, and specific granules of polymorphonuclear leukocytes (PMNs)(3). Lactoferrin is found mainly in the oral cavity where it can come into direct contact with pathogens such as viruses, bacteria, etc. Lactoferrin directly inhibits viruses by binding to viral receptor sites, thus preventing the virus from infecting healthy cells. Lactoferrin has a direct bacteriacidal function to certain bacteria such as *Streptococcus mutans, Vibrio cholerae, Escherichia coli, Actinobacillus actinomycetemcomitans,* and *Legionella pneumophila (2,3,4).* Also, it has a bacteriostatic effect that deprives iron-requiring bacteria of this essential growth nutrient (4). Lactoferrin is also considered an antioxidant that scavenges free iron, helping to prevent uncontrolled iron based free radical reactions, thus protecting certain cells from peroxidation. (2)

Principal of the Assay

The Human Lactoferrin ELISA kit is designed for detection of human plasma, serum, urine, saliva, milk and cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay technique, which measures lactoferrin in less than 4 hours. A polyclonal antibody specific for lactoferrin has been pre-coated onto a microplate. Lactoferrin in standards and samples is sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for lactoferrin, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- **Prepare all reagents (working diluent buffer, wash buffer, standards, biotinylated-antibody, and SP conjugate) as instructed, prior to running the assay.**
- **Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this protocol. However, the user should determine the optimal dilution factor.**
- **Spin down the SP conjugate vial and the biotinylated-antibody vial before opening and using contents.**
- This kit is for research use only.
- The kit should not be used beyond the expiration date.
- The Stop Solution is an acid solution.

Reagents

- **Human Lactoferrin Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against human lactoferrin.
- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **Human Lactoferrin Standard:** Human Lactoferrin in a buffered protein base (640 ng, lyophilized).
- **Biotinylated Lactoferrin Antibody (100x):** A 100-fold concentrated biotinylated polyclonal antibody against human lactoferrin (80 µl).
- **MIX Diluent Concentrate (10x)**: A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x)**: A 20-fold concentrated buffered surfactant (30 ml, 2 bottles).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate**: A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution**: A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Store components of the kit at 2-8^{0}C or -20^{0}C upon arrival up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody at -20^{0}C
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8^{0}C
- Opened unused microplate wells may be returned to the foil pouch with the desiccant packs. Reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.
- Diluent (1x) may be stored for up to 1 month at 2-8^{0}C.
- Store Standard at 2-8^{0}C before reconstituting with Diluent and at -20^{0}C after reconstituting with Diluent.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm
- Pipettes (1-20 µl, 20-200 µl, 200-1000 µl and multiple channel)
- Deionized or distilled reagent grade water

Sample Collection, Preparation and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 2000 x g for 10 minutes. Dilute samples 1:50 into MIX Diluent. The undiluted samples can be stored at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles. (EDTA or Heparin can also be used as anticoagulant.)
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2000 x g for 10 minutes. Remove serum and dilute samples 1:50 into MIX Diluent. Store serum at -20^{0}C or below. Avoid repeated freeze-thaw cycles.
- **Cell Culture Supernatants:** Centrifuge cell culture media at 2000 x g for 10 minutes to remove debris. Collect supernatants and assay. Store the remaining samples at -20^{0}C or below. Avoid repeated freeze-thaw cycles.
- **Urine:** Collect urine using sample pot. Centrifuge samples at 600 x g for 10 minutes. Dilute samples 1:10 into MIX Diluent and assay. Store samples at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Saliva:** Collect saliva to sample tube. Centrifuge samples at 600 x g for 10 minutes. Dilute samples 1:1000 into MIX Diluent and assay. The undiluted samples can be stored at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

- **Milk:** Centrifuge samples at 600 x g for 10 minutes and assay. Dilute samples 1:100000 into MIX Diluent. Store samples at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **MIX Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the MIX Diluent 1:10 with reagent grade water. Store for up to 1 month at 2-8^{0}C.
- **Standard Curve:** Reconstitute the 640 ng of Lactoferrin Standard with 4 ml of MIX Diluent to generate a stock solution of 160 ng/ml. Allow the stock solution to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare the standard solution by diluting the stock solution (160 ng/ml) 1:4 with MIX Diluent to produce 40 ng/ml. Prepare duplicate or triplicate points by serially diluting the standard (40 ng/ml) 1:2 using equal volume of MIX Diluent to give 20, 10, 5, 2.5, 1.25 and 0.625 ng/ml solutions. MIX Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at < -20^{0}C.

Standard Point	Dilution	[Lactoferrin] (ng/ml)
P1	1 part Stock (160 ng/ml) + 3 parts MIX Diluent	40.000
P2	1 part P1 + 1 part MIX Diluent	20.000
P3	1 part P2 + 1 part MIX Diluent	10.000
P4	1 part P3 + 1 part MIX Diluent	5.000
P5	1 part P4 + 1 part MIX Diluent	2.500
P6	1 part P5 + 1 part MIX Diluent	1.250
P7	1 part P6 + 1 part MIX Diluent	0.625
P8	MIX Diluent	0.000

- **Biotinylated Lactoferrin Antibody (100x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:100 with MIX Diluent. Any remaining solution should be frozen at -20^{0}C.
- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with MIX Diluent. Any remaining solution should be frozen at -20^{0}C.

Assay Procedure

- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30^{0}C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of Standard or sample per well. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
  If using a machine wash six times with 300 µl of Wash Buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
- Add 50 µl of Biotinylated Lactoferrin Antibody to each well and incubate for one hour.
- Wash the microplate as described above.
- Add 50 µl of Streptavidin-Peroxidase Conjugate per well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.

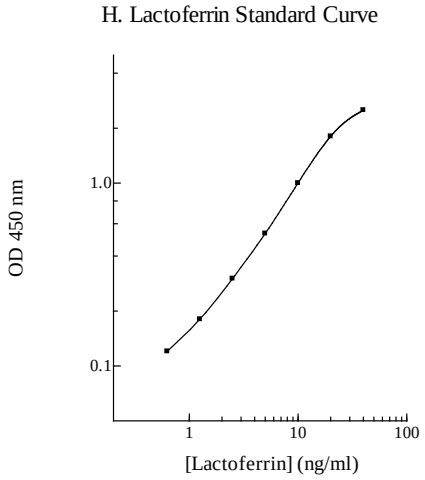
- Add 50 µl of Chromogen Substrate per well and incubate for about 15 minutes or till the optimal blue color density develops. Gently tap the plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 µl of Stop Solution to each well. The color will change from blue to yellow.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a Standard Curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.



Performance Characteristics

- The minimum detectable dose of Lactoferrin is typically 0.6 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 4.8 % and 7.3 % respectively.

Linearity

	Average Percentage of Expected Value	
Sample Dilution	**Plasma**	**Serum**
1:25	93%	91%
1:50	101%	99%
1:100	105%	102%

	Average Percentage of Expected Value
Sample Dilution	**Urine**
1:5	95%
1:10	99%
1:20	104%

Recovery

Standard Added Value	2 – 20 ng/ml
Recovery %	86-115%
Average Recovery %	98 %

Cross-Reactivity

Species	% Cross Reactivity
Canine	1%
Bovine	0.5%
Monkey	10%
Mouse	0.5%
Rat	0.5%
Swine	1%
Rabbit	1%

- If cell culture supernatants contains 10% FBS, the minimum detectable dose of human lactoferrin will be 1 ng/ml.