# Human Lactoferrin
# ELISA Kit

CE IVD

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## Assay Summary

**Step 1**. Add 50 µl of Standard or Sample per well.
Incubate 2 hours.

**Step 2.** Wash, then add 50 µl of Biotinylated Antibody per well.
Incubate 1 hour.

**Step 3**. Wash, then add 50 µl of SP Conjugate per well.
Incubate 30 minutes.

**Step 4.** Wash, then add 50 µl of Chromogen Substrate per well.
Incubate 15 minutes.

**Step 5.** Add 50 µl of Stop Solution per well.
Read at 450 nm immediately.

## Symbol Key

For In Vitro Diagnostic Use


Expiration Date


Temperature Requirements


Consult Instructions for Use

Catalog Number

Lot Number


Manufacturer

Authorized Representative

For any questions regarding troubleshooting or performing the assay, please contact our support team at support@assaypro.com.

Thank you for choosing Assaypro.

# Assay Template

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

# AssayMax Human Lactoferrin ELISA Kit

REF EL2011-4 30 APR 2017

***Sample protocol for reference use only***

*Read package protocol completely before using product. Follow instructions carefully when performing tests; failure to follow protocol may result in inaccurate results.*

## Introduction

Lactoferrin is an 80 kDa iron-binding glycoprotein produced by many exocrine glands with a major constituent in the secondary granules of neutrophilic 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. Lactoferrin 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 bactericidal function to certain bacteria such as *Streptococcus mutans, Vibrio cholerae, Escherichia coli, Actinobacillus actinomycetemcomitans,* and *Legionella pneumophila* (2-4). 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).

## Intended Use

IVD

The AssayMax Lactoferrin ELISA Kit is an enzyme immunoassay for the quantitative determination of lactoferrin to be used in conjunction with clinical evaluation and patient risk assessment. Lactoferrin can be used as an aid in predicting risk for inflammation. This assay is intended for *in vitro diagnostic use.*

## Principle of the Assay

The AssayMax Human Lactoferrin ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for detection of human lactoferrin in **plasma, serum, urine, saliva, milk, CSF, and cell culture samples**. 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 96-well microplate with removable strips. 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, wash buffer, standard, biotinylated antibody, and SP conjugate) as instructed, prior to running the assay.
- Spin down the SP conjugate vial and the biotinylated antibody vial before opening and using contents.
- Prepare all samples prior to running the assay. The dilution factors for samples are suggested in this protocol. However, the user should determine the optimal dilution factor.
- Intended for in vitro diagnostic use and should not be used as the sole testing method in therapeutic procedures.
- The Stop Solution is an acidic solution.
- All reagents should be considered potentially hazardous. It is recommended that this product is handled only by professionals trained in laboratory techniques and is used in accordance with the principles of good laboratory practice.
- The device contains materials of human and animal origin and should be handled as a potential transmitter of disease. All human source material have tested negative for antibodies to HIV, HbsAg and HCV. However, no test method can offer complete assurance that infectious agents are not present. PPE should be used when handling any patient sera or serum based products.
- Any material in contact with potentially infectious material must be disinfected or discarded into a biohazard container. Dispose of reagents and specimens as clinical waste. Any unused reagents should be flushed away with copious amounts of water. Disposal must be performed in accordance with local legislation.

## 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 precut, 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 (280 ng, lyophilized).
- **Biotinylated Human Lactoferrin Antibody (50x):** A 50-fold concentrated biotinylated polyclonal antibody against human lactoferrin (120 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

- Upon arrival, immediately store unopened components of the kit at recommended temperatures.

| Component | Before opening or reconstituting | After opening or reconstituting |
|---|---|---|
| Microplate | +2-8°C | +2-8°C |
| Standard | +2-8°C | -20°C |
| Biotinylated Antibody | -20°C | -20°C |
| SP Conjugate | -20°C | -20°C |
| MIX Diluent (1x) | +2-8°C | +2-8°C |
| Wash Buffer (1x) | +2-8°C | +2-8°C |
| Chromogen Substrate | +2-8°C | +2-8°C |
| Stop Solution | +2-8°C | +2-8°C |
| MIX Diluent Concentrate (10x) | +2-8°C | +2-8°C |
| Wash Buffer Concentrate (20x) | +2-8°C | +2-8°C |

- **Microplate**: Unused wells may be returned to the foil pouch with the desiccant packs and resealed. May also be stored for up to 30 days in a vacuum desiccator.

## Expiration Date

- Kit should not be used beyond the expiration date.

| | Before opening or reconstituting | After opening or reconstituting |
|---|---|---|
| All Reagents | 30 APR 2017 | Use within 30 days |

## Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm
- Program with statistical calculator to perform linear regression analysis
- Device for delivery of wash buffer solution (e.g. automatic wash system)
- Containers necessary for preparation of reagents
- Pipettes (1-20 µl, 20-200 µl, 200-1000 µl, and multiple channel pipette)
- Disposable tips and a multichannel micropipette reservoir
- 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 3000 x *g* for 10 minutes. Dilute samples 1:50 into MIX Diluent and assay. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles (EDTA or Heparin can also be used as an anticoagulant).
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3000 x *g* for 10 minutes, and remove serum. Dilute samples 1:50 into MIX Diluent and assay. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Cell Culture Supernatants:** Centrifuge cell culture media at 3000 x *g* for 10 minutes to remove debris. Collect supernatants and assay. Store the remaining samples at -20°C or below. Avoid repeated freeze-thaw cycles.
- **Urine:** Collect urine using sample pot. Centrifuge samples at 800 x *g* for 10 minutes and assay. If necessary, dilute samples within the range of 1:2 to 1:10 into MIX Diluent, and assay. The undiluted samples can be

stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

- **Saliva:** Collect saliva to sample tube. Centrifuge samples at 800 x *g* for 10 minutes. Dilute samples 1:1000 into MIX Diluent and assay. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Milk:** Centrifuge samples at 800 x *g* for 10 minutes. Dilute samples 1:100000 into MIX Diluent and assay. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **CSF:** Collect cerebrospinal fluid (CSF) using sample pot. Centrifuge samples at 3000 x *g* for 10 minutes. Dilute samples 1:10 into MIX Diluent and assay. The undiluted samples can be stored at -80°C for up to 3 months. Avoid repeated freeze-thaw cycles.

***Refer to Sample Dilution Guidelines below for further instruction.***

| **Guidelines for Dilutions of 1:100 or Greater**<br>(*for reference only; please follow the protocol for specific dilution suggested)* | |
|---|---|
| 1:100 | 1:10000 |
| A) 4 ul sample: 396 µl buffer(100x)<br>= 100 fold dilution<br><br>*Assuming the needed volume is less than or equal to 400 µl.* | A) 4 µl sample : 396 µl buffer (100x)<br>B) 4 µl of A : 396 µl buffer (100x)<br>= 10000 fold dilution<br>*Assuming the needed volume is less than or equal to 400 µl.* |
| 1:1000 | 1:100000 |
| A) 4 µl sample : 396 µl buffer (100x)<br>B) 24 µl of A : 216 µl buffer (10x)<br>= 1000 fold dilution<br><br>*Assuming the needed volume is less than or equal to 240 µl.* | A) 4 µl sample : 396 µl buffer (100x)<br>B) 4 µl of A : 396 µl buffer (100x)<br>C) 24 µl of B : 216 µl buffer (10x)<br>= 100000 fold dilution<br>*Assuming the needed volume is less than or equal to 240 µl.* |

## 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 Concentrate 1:10 with reagent grade water. Store for up to 30 days at 2-8°C.
- **Standard Curve:** Reconstitute the 280 ng of Human Lactoferrin Standard with 3.5 ml of MIX Diluent to generate an 80 ng/ml standard stock solution. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate points by serially

diluting the standard stock solution (80 ng/ml) 1:2 using equal volume of MIX Diluent to produce 40, 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°C and used within 30 days.

| Standard Point | Dilution | [Lactoferrin] (ng/ml) |
|---|---|---|
| P1 | 1 part Standard (80 ng/ml) + 1 part MIX Diluent | 40.00 |
| P2 | 1 part P1 + 1 part MIX Diluent | 20.00 |
| P3 | 1 part P2 + 1 part MIX Diluent | 10.00 |
| 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 Human Lactoferrin Antibody (50x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:50 with MIX Diluent. Any remaining solution should be frozen at -20°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°C.

## Assay Procedure

- Prepare all reagents, standard solutions, and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-25°C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccants inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of Human Lactoferrin Standard or sample per well. Cover wells with a sealing tape and incubate for 2 hours. Start the timer after the last addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If using a machine, wash six times with 300 µl of Wash Buffer and then invert the plate, decanting the contents; hit 4-5 times on absorbent material to completely remove the liquid.

- Add 50 μl of Biotinylated Human Lactoferrin Antibody to each well and incubate for 1 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 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 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.
- It is suggested to use a control sample of known concentration with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid. Each testing facility should establish its own acceptable range.

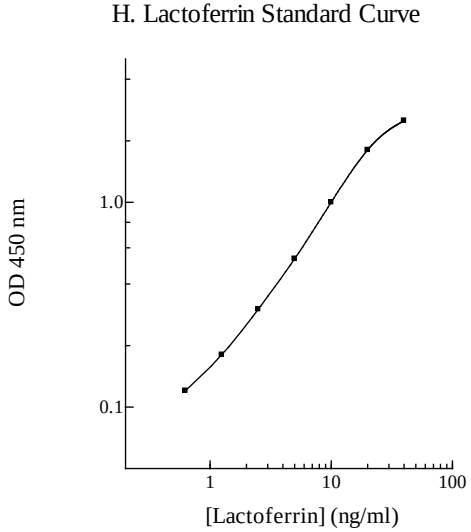
## Typical Data

- The typical data is provided for reference only. Individual laboratory means may vary from the values listed. Variations between laboratories may be caused by technique differences.

| Standard Point | ng/ml | OD | Average OD |
|---|---|---|---|
| P1 | 40.00 | 2.150<br>2.144 | 2.147 |
| P2 | 20.00 | 1.715<br>1.711 | 1.713 |
| P3 | 10.00 | 1.142<br>1.147 | 1.145 |
| P4 | 5.000 | 0.700<br>0.704 | 0.702 |
| P5 | 2.500 | 0.370<br>0.360 | 0.365 |
| P6 | 1.250 | 0.202<br>0.200 | 0.201 |
| P7 | 0.625 | 0.128<br>0.114 | 0.121 |
| P8 | 0.000 | 0.049<br>0.039 | 0.044 |
| **Sample: Pool Normal, Sodium Citrate Plasma (50x)** | | 0.741<br>0.737 | 0.739 |

## Standard Curve

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



## Reference Value

- Normal human lactoferrin plasma levels range from 150 to 850 ng/ml.
- Human plasma and serum samples from healthy adults were tested (n=40). On average, lactoferrin level was 330 ng/ml.

| Sample | n | Average Value (ng/ml) |
|---|---|---|
| Human Pooled Normal Plasma | 10 | 336 |
| Human Normal Plasma | 20 | 285 |
| Human Pooled Normal Serum | 10 | 371 |

## Performance Characteristics

- The minimum detectable dose of lactoferrin as calculated by 2SD from the mean of a zero standard was established to be 0.35 ng/ml.
- Intra-assay precision was determined by testing replicates of three plasma samples in one assay.
- Inter-assay precision was determined by testing three plasma samples in twenty assays.

| | Intra-Assay Precision | | | Inter-Assay Precision | | |
|---|---|---|---|---|---|---|
| Sample | 1 | 2 | 3 | 1 | 2 | 3 |
| n | 20 | 20 | 20 | 20 | 20 | 20 |
| CV (%) | 3.3% | 3.8% | 3.9% | 8.4% | 8.6% | 8.4% |
| Average CV (%) | 3.7% | | | 8.5% | | |

## Spiking Recovery

- Recovery was determined by spiking two plasma samples with different lactoferrin concentrations.

| Sample | Unspiked Sample (ng/ml) | Spike (ng/ml) | Expected | Observed | Recovery (%) |
|---|---|---|---|---|---|
| 1 | 2.0 | 10.0 | 12.0 | 12.5 | 104% |
| | | 5.0 | 7.0 | 8.1 | 116% |
| | | 2.5 | 4.5 | 4.8% | 107% |
| 2 | 5.0 | 10.0 | 15.0 | 13.2 | 88% |
| | | 5.0 | 10.0 | 10.3 | 103% |
| | | 2.5 | 7.5 | 7.2 | 96% |
| **Average Recovery (%)** | | | | | 102% |

## Linearity

- Plasma and serum samples were serially-diluted to test for linearity.

| **Average Percentage of Expected Value (%)** | | |
|---|---|---|
| Sample Dilution | Plasma | Serum |
| 1:25 | 92% | 89% |
| 1:50 | 99% | 98% |
| 1:100 | 105% | 104% |

## Cross-Reactivity

| **Species** | **Cross Reactivity (%)** |
|---|---|
| Canine | None |
| Bovine | None |
| Monkey | 10% |
| Mouse | None |
| Rat | None |
| Swine | None |
| Rabbit | None |

## Troubleshooting

| **Issue** | **Causes** | **Course of Action** |
|---|---|---|
| **Low Precision** | Use of expired components | • Check the expiration date listed before use.<br>• Do not interchange components from different lots. |
| | Improper wash step | • Check that the correct wash buffer is being used.<br>• Check that all wells are empty after aspiration.<br>• Check that the microplate washer is dispensing properly.<br>• If washing by pipette, check for proper pipetting technique. |
| | Splashing of reagents while loading wells | • Pipette properly in a controlled and careful manner. |

| | | |
|---|---|---|
| | Inconsistent volumes loaded into wells | • Pipette properly in a controlled and careful manner.<br>• Check pipette calibration.<br>• Check pipette for proper performance. |
| | Insufficient mixing of reagent dilutions | • Thoroughly agitate the lyophilized components after reconstitution.<br>• Thoroughly mix dilutions. |
| | Improperly sealed microplate | • Check the microplate pouch for proper sealing.<br>• Check that the microplate pouch has no punctures.<br>• Check that three desiccants are inside the microplate pouch prior to sealing. |
| **Unexpectedly Low or High Signal Intensity** | Microplate was left unattended between steps | • Each step of the procedure should be performed uninterrupted. |
| | Omission of step | • Consult the provided procedure for complete list of steps. |
| | Steps performed in incorrect order | • Consult the provided procedure for the correct order. |
| | Insufficient amount of reagents added to wells | • Check pipette calibration.<br>• Check pipette for proper performance. |
| | Wash step was skipped | • Consult the provided procedure for all wash steps. |
| | Improper wash buffer | • Check that the correct wash buffer is being used. |
| | Improper reagent preparation | • Consult reagent preparation section for the correct dilutions of all reagents. |
| | Insufficient or prolonged incubation periods | • Consult the provided procedure for correct incubation time. |
| **Deficient Standard Curve Fit** | Non-optimal sample dilution | • Sandwich ELISA: If samples generate OD values higher than the highest standard point (P1), dilute samples further and repeat the assay.<br>• Competitive ELISA: If samples generate OD values lower than the highest standard point (P1), dilute samples further and repeat the assay.<br>• User should determine the optimal dilution factor for samples. |
| | Contamination of reagents | • A new tip must be used for each addition of different samples or reagents during the assay procedure. |
| | Contents of wells evaporated | • Verify that the sealing film is firmly in place before placing the assay in the incubator or at room temperature. |
| | Improper pipetting | • Pipette properly in a controlled and careful manner.<br>• Check pipette calibration.<br>• Check pipette for proper performance. |
| | Insufficient mixing of reagent dilutions | • Thoroughly agitate the lyophilized components after reconstitution.<br>• Thoroughly mix dilutions. |

## References


(1) Naot, D. *et al.* (2005) *Clinical Medicine & Research*. Vol 3, No. 2: 93-101
(2) Brink, W. (October 2000) LE Magazine.
(3) Yamauchi, K. *et al* (1993) *Infection and Immunity.* Vol 61, No 2, p.719-728
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