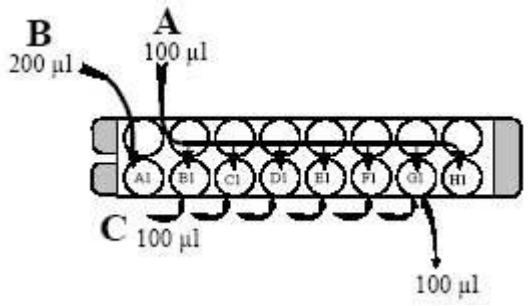
Revision	06-May-2009 RFH
Form	96 Tests
Format	96-well plate
Detection method	Colorimetric
Species	human
Storage	Upon arrival store the entire contents of the kit at 4° C.
Background	Human lactoferrin (LTF) is an 80 kDa glycoprotein found in many mucosal secretions. Blood LTF is secreted from neutrophils and its plasma concentration is considered to be an indicator of neutrophil turnover. LTF's bactericidal properties are attributed to its strong iron binding capacity. During inflammation, LTF is often released into the extracellular medium from secondary granules of neutrophils. Therefore, its extracellular concentration can be used as an index of neutrophil activation, especially in blood samples containing anti-myeloperoxidase antibodies.
Principles of the assay	The lactoferrin assay kit uses an enzyme-linked immunoassay (ELISA) method. Samples are incubated in the wells of a divided microplate that have been coated with a primary monoclonal antibody to LTF. The presence of LTF is detected using a biotinylated-monoclonal antibody to LTF. The final step in the assay uses amplification based on biotin-avidin coupling where avidin has been covalently linked to horseradish peroxidase (HRP). The amount of LTF is measured colorimetrically upon addition of the HRP substrate *o*-phenylenediamine (OPD).

Materials provided	• **Sample Diluting Buffer (Kit Component No. KP1801):** 3 bottles, 25 ml each, phosphate buffer pH 7.4, with NaCl, bovine serum albumin (BSA), Tween®-20 detergent, and 0.01% thimerosal. This solution is used to dilute biological samples • **LTF Standard (Kit Component No. KP1802):** 1 vial, 300 ng ± 5 ng of purified LTF, lyophilized. • **Washing Buffer (20X) (Kit Component No. KP1803):** 1 bottle, 100 ml, 1 M Tris-HCl buffer, pH 7.8, containing 3 M NaCl, 2% Tween®-20 detergent, and 0.01% merthiolate. • **Anti-LTF Solution (Kit Component No. KP1804):** 1 vial, 75 µl, Biotinylated monoclonal antibody to LTF in 20 mM phosphate buffer, pH 7.4, containing 150 mM NaCl, 2 mg/ml BSA, 25% glycerol, and 0.01% merthiolate. • **Avidin-HRP Solution (Kit Component No. KP1805):** 1 vial, 75 µl, Avidin coupled to horseradish peroxidase in 50 mM Tris-HCl buffer, pH 8.0, containing 20 mg/ml BSA and 0.01% merthiolate. • **Diluting Buffer (Kit Component No. KP1806):** 3 vials, 8 ml each, 20 mM phosphate buffer, pH 7.4, with 150 mM NaCl, 20 mg/ml BSA, 0.1% Tween®-20 detergent, and 0.01% merthiolate. • **OPD Diluting Buffer (Kit Component No. KP1807):** 1 bottle, 20 ml, 100 mM citrate buffer, pH 5.5, containing 0.012% H_2O_2, and 0.01 % merthiolate. • **Stop Solution (Kit Component No. KP1808):** 1 bottle, 20 ml, 1 M H_2SO_4. • **OPD Substrate (Kit Component No. KP1809):** 4 tablets, each tablet contains 20 mg of OPD. • **Microplates (Kit Component No. KP1810):** One 96-well microplate supplied as 6x16-well strips, with frame.
Materials Required but not provided	• Deionized water. • Test tubes and beakers. • Precision pipettes adjustable in the range of 0.1 to 1 ml. • Automatic pipetters (100 and 50 µl) to add reagents into the plate wells. • Incubator at 37° ± 1° C. • Spectrophotometric microplate reader capable of measuring absorbance at 450 nm.
Precautions and recommendations	• Do not smoke, eat or drink in areas where reagents and samples are handled. • Do not pipette by mouth.

	• Use pipettes with disposable tips to avoid bacterial contamination. • Wear disposable gloves when handling reagents and samples. • In case of accidental exposure of skin, eyes, or mucous membranes to Stop Solution, wash the exposed area thoroughly with water for 15 min. • Human LTF was purified from human milk shown to be negative for HIV and HBV. LTF was heat-inactivated and treated by detergent in order to inactivate HIV. Handle LTF Standard, solution with the same precautions that are usually required for human blood products.
Preparation	Note: If necessary, dilute the samples with sample diluting buffer. Add 100 µl of sample per well. **1. Blood plasma** Draw venous blood into a glass test tube that contains EDTA as an anticoagulant. Whole blood should be stored at 4° C. Centrifuge whole blood at 3000 x*g* for 10 min at 4° C and recover the resulting plasma supernatant. Plasma samples can be stored at 4° C for up to 24 h. For longer storage, freeze the samples at -20° C. Avoid freeze/thaw cycles. Ideally, plasma separation should be performed within 6 h. Plasma samples should be diluted 1:40 (v/v) in sample diluting buffer, for LTF values in the range 45-to-3000 ng/ml. With higher concentrations of LTF, greater dilutions will be required. Note: Heparin may interfere with LTF measurements. **2. Urine** Urine should be recovered in clean flasks. Samples that are turbid, due to the presence of cellular or crystalline residues, should be clarified by centrifugation at 1000 x *g* for 10 minutes. Such samples can be stored at 4° C for up to 3 days, with or without pH adjustment. If the measured concentration of LTF is greater than 100 ng/ml in urine, dilute with sample diluting buffer. Otherwise, use undiluted sample. **3. Broncho-alveolar lavage (BAL)** If the measured concentration of LTF is greater than 100 ng/ml, dilute with sample diluting buffer. Otherwise, use undiluted sample. **4. Cerebrospinal fluid** If the measured concentration of LTF is greater than 100 ng/ml, dilute with sample diluting buffer. Otherwise, use undiluted sample. **5. Supernatants after centrifugation of cell cultures** In a number of experimental conditions, LTF is released from human cells in culture. The Lactoferrin ELISA Kit can be used to assay LTF in culture medium that contains up to 20% fetal calf serum (FCS). However, if the measured concentration of LTF is greater than 100 ng/ml, samples

	should be diluted in sample diluting buffer
Reagent preparation	All solutions must be kept at room temperature for 30 min before use. • **Sample Diluting Buffer:** The original solution is ready for use. • **LTF Standard:** Prepare a solution of LTF standard by adding 3.0 ml of Sample Diluting Buffer to the lyophilized protein. Exercise caution when opening the original vacuum-sealed bottle. The resulting solution may be stored at 4° C for up to 3 days. For longer term storage, aliquot and store at -70° C. To prepare a standard curve pipette 100 µl of sample diluting buffer in wells B1 through H1, as shown below. Well H1 will serve as the negative control. Pipette 200 µl of LTF standard, solution in well A1 as shown. Perform serial dilutions by transferring 100 µl from one well into the next one as shown. After each transfer, mix thoroughly using 4 suctions at each step with a pipette. Mix slowly in order to avoid foaming. Use a fresh pipette tip for each well. Cover the wells with adhesive paper and incubate at 37° C for 1 h. Figure 1: Organization of standards and samples on microplates.  • **Washing Solution (20x):** The original buffer should be diluted 1:20 prior to use. Once prepared, the diluted washing buffer (1x) can be stored for up to 5 days at room temperature. Table 1: Washing solution dilutions | Number of 8-well-strips	Required volume of washing buffer	Volume of concentrated washing buffer (20x)	Volume of deionized water to be added
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4	250 ml	12.5 ml	237.5 ml
8	500 ml	25 ml	475 ml
12	1000 ml	50 ml	950 ml	 • **Biotinylated anti-LTF Solution:** This reagent must be diluted ~1:250 in Diluting Buffer prior to use.

	Table 2: Anti-LTF solution dilutions 	Number of 8-well-strips	Volume of anti-LTF solution	Volume of diluting buffer
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4	16 µl	4 ml
8	32 µl	8 ml
12	48 µl	12 ml | • **Avidin-HRP Solution:** This reagent must be diluted ~1:250 in Diluting Buffer prior to use. Table 3: Avidin-HRP solution dilutions | Number of 8-well-strips	Volume of avidin-HRP solution	Volume of diluting buffer
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4	16 µl	4 ml
8	32 µl	8 ml
12	48 µl	12 ml | • **OPD Solution:** The OPD solution should be prepared 5 min prior to use. Dissolve the required number of OPD tablets in OPD Diluting Buffer. Tablets can be dissolved more quickly using a magnetic stirrer. Table 4: OPD solution dilutions | Number of 8-well-strips	Number of OPD tablets	Volume of OPD diluting buffer
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4	1	5 ml
8	2	10 ml
12	3	15 ml	 **1. Organization of samples and standards on microplates** Unpack the exact number of strips required for assay and set them on the frame. Determine the layout of standard and samples (see example below). Figure 2: Standard Curve Preparation
Detailed protocol	

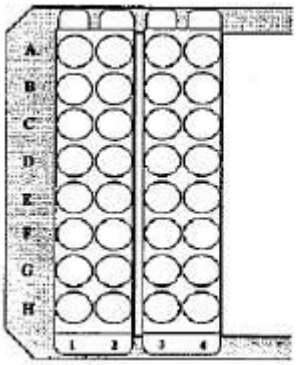
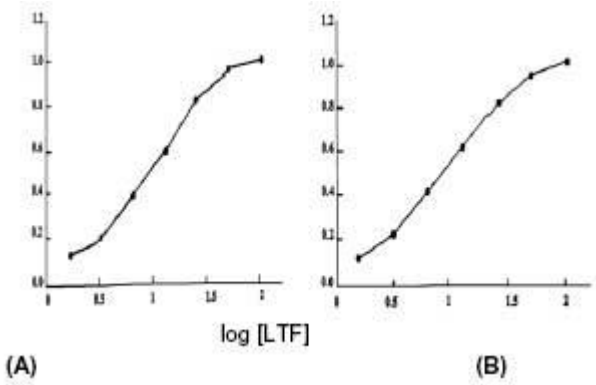
	 **LEGEND** A1: LTF Standard, 100 ng/ml B1: LTF Standard, 50 ng/ml C1: LTF Standard, 25 ng/ml D1: LTF Standard, 12.5 ng/ml E1: LTF Standard, 6.2 ng/ml F1: LTF Standard, 3.1 ng/ml G1: LTF Standard, 1.6 ng/ml H1: Negative Control A2 through H4: Samples 2. Anti-LTF incubation Prepare the required volume of diluted biotinylated anti-LTF. Empty the microplate. Wash each well 5 times with washing buffer diluted 1:20. Drain the residual washing solution on absorbing paper. Add 100 µl of diluted biotinylated anti-LTF to each well. Cover the wells with adhesive paper and incubate at 37° C for 1 h. 3. Avidin-HRP incubation Prepare the required volume of diluted avidin-horseradish peroxidase solution. Empty the microplate. Wash each well 5 times with washing buffer diluted 1:20. Drain the residual washing solution on absorbing paper. Add 100 µl of diluted avidin-horseradish peroxidase solution into each well. Cover the wells with adhesive paper and incubate at 37° C for 15 min. 4. Colorimetric measurement Prepare the required volume of OPD solution. Empty the microplate. Wash each well 5 times with Washing Buffer diluted 1:20. Drain the residual washing solution on absorbing paper. Add 100 µl of prepared OPD solution to each well. Cover the wells with adhesive paper and incubate at 37° C for about 5-10 min until the absorbance of well number 1 (100 ng/ml) reaches approximately 1-1.5. Add 50 µl of Stop Solution into each well. Read the absorbance at 450 nm.
Calculations	The standard curve is obtained by plotting the absorbance at 450 nm as a function of the logarithm of standard LTF concentrations in ng/ml. Sample values are then obtained by interpolation or more accurately by using a third-order polynomial regression. The two modes of standard curve plotting are illustrated in

	Figure 3. Figure 3: Example of standard curves:  (A) Third order polynomial regression (B) Interpolation.
Sensitivity	>1 ng/ml
Sensitivity Notes	From 36 repeated measurements performed on sample blank with the same microplate within a single day, the detection limit of the assay was found to be 1 ng/ml.
Protocol Summary	**Quick Reference Procedure for Lactoferrin ELISA Kit Material Preparation Protocol** 1. Place solutions at room temperature 30 min before assay. 2. Prepare standard by adding 3 ml of diluting buffer to LTF standard. 3. Dilute wash buffer solution 1:20 with water. 4. Dilute anti-LTF 1:250 with diluting buffer. 5. Dilute avidin-alkaline phosphatase solution 1:250 with diluting buffer. 6. Dilute samples if necessary. 7. Prepare OPD solution 5 min before use. **ELISA Protocol** 1. Add 100 µl sample/standard to each well. 2. Cover plate and incubate for 1 h at 37° C. 3. Wash wells 5 times with washing buffer. 4. Add 100 µl diluted anti-LTF solution. 5. Cover plate and incubate for 1 h at 37° C. 6. Wash wells 5 times with washing buffer. 7. Add 100 µl diluted avidin-HRP solution. 8. Cover plate and incubate for 15 min at 37° C. 9. Wash wells 5 times with washing buffer. 10. Add 100 µl OPD solution. 11. Cover plate and incubate 5 to 10 min at 37° C. 12. Add 50 µl of stop solution. 13. Mix and read the absorbance at 450 nm. 14. Plot absorbance as a function of LTF concentration.
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