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CERTIFICATE OF ANALYSIS

LTB_4 ELISA Kit

Ordering Code: EHLTB4 **Lot Number:** OF187571

Kit Storage: -20°C in a manual defrost freezer. Avoid repeated freeze-thaw cycles.

Product: A competitive immunoassay for the quantitative determination of Leukotriene B_4 in biological fluids. The assay is based on the competition between LTB_4 in the standard or sample and Alkaline Phosphatase conjugated LTB_4 (LTB_4-AP) for a limited amount of LTB_4 monoclonal antibody bound to an Anti-Rabbit IgG precoated 96-well plate. As the concentration of LTB_4 in the sample increases, the amount of LTB_4-AP captured by the coating antibody decreases. Thus, there is an inverse relationship between optical density (OD) and the amount of analyte in the sample.

LTB_4 ELISA KIT COMPONENTS

***Allow all Kit Components to come to room temperature for at least 30 minutes prior to opening.*

Description	Size	Form	Component Usage	Lot
Anti-Rabbit IgG Plate	1 plate	A 96-well strip plate coated with goat antibody specific to rabbit IgG.	§ Unused wells must be kept desiccated at 2 - 8°C in the sealed foil bag. § Use wells in the frame provided.	OF187571B
Reagent Diluent	30 ml	Tris Buffered Saline containing proteins and a preservative.	Ready to Use	OF187571E
LTB_4 Antibody	6 ml	A yellow solution of a monoclonal antibody to LTB_4.	Ready to Use	OF187571G
LTB_4-AP Conjugate	6 ml	A blue solution of LTB_4 conjugated to alkaline phosphatase.	Ready to Use **Store conjugate at -20°C. Aliquot to avoid repeated freeze-thaw cycles.**	OF187571A
LTB_4 Standard	0.5 ml	A solution of 120,000 pg/ml LTB_4.	§ Label five 12x75 mm glass tubes #1 - #5. § Pipet 900 µl of Standard Diluent (Reagent Diluent or Tissue Culture Media) into Tube #1. § Pipet 750 µl of Standard Diluent into tubes #2 - #5. § Add 100 µl of stock LTB_4 standard to tube #1 and vortex. § Add 250 µl from tube #1 to tube #2 and vortex. § Add 250 µl from tube #2 to tube #3 and vortex. § Continue this for tubes #4 and #5. § The concentration of LTB_4 in tubes #1 - #5 will be 12,000, 3,000, 750,188 and 46.9 pg/ml, respectively. § Use diluted standards within 60 minutes. § **Store concentrated standard at -20°C. Aliquot to avoid repeated freeze-thaw cycles.**	OF187571C
10X Wash Buffer	30 ml	Tris Buffered Saline containing detergents and a preservative.	§ Dilute 10 ml of the 10X Wash Buffer with 90 ml of deionized water. § 1X Wash Buffer can be stored for up to 3 months at room temperature.	OF187571F
Substrate Solution	20 ml	A solution of p-nitrophenyl phosphate in buffer.	Ready to Use	OF187571D
Stop Solution	6 ml	A solution of trisodium phosphate in water.	Ready to Use	OF187571H
Plate Sealer	1 each		Ready to Use	
LTB_4 Assay Layout Sheet	1 each		Ready to Use	

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LTB_4 ELISA KIT Procedural Notes

1. Materials needed but not supplied with kit include: deionized or distilled water; precision pipets for volumes between 5 µl and 1,000 µl; repeater pipets for dispensing 50 µl and 200 µl; disposable beaker for diluting buffer concentrates; graduated cylinders; a 37°C incubator, a microplate shaker; absorbent paper for blotting; and a microplate reader capable of reading at 405 nm, preferable with correction between 570 and 590 nm.
2. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
3. Pre-rinse the pipet tip with the reagent, use fresh pipet tips for each sample, standard or reagent.
4. Care must be taken to minimize contamination by endogenous alkaline phosphatase (AP). Contaminating AP activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.
5. Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.

Sample Handling

The LTB_4 ELISA is compatible with LTB_4 samples in a wide range of matrices after dilution in Reagent Diluent. Tissue culture media and human urine is recommended to be used neat. Human saliva is recommended at a >1:4 dilution. Porcine EDTA Plasma is recommended to be diluted 1:2 - 1:4. Verification of the appropriate dilution is required for the individual sample type. Samples containing rabbit IgG may interfere with the assay.

Samples in the majority of tissue culture media, including those containing fetal bovine serum can also be used in this assay, provided the standards have been diluted into the tissue culture media instead of Reagent Diluent. There will be a small change in binding associated with running the standards and samples in media. Standard curves should be run in the appropriate matrix. For tissue, urine and plasma samples, prostaglandin synthetase inhibitors such as indomethacin or meclofenamic acid at concentrations up to 10 µg/ml should be added to the tissue homogenate, urine and plasma samples.
Some samples normally have very low levels of LTB_4 present and extraction may be necessary for accurate measurement.

Extraction Procedure Example

1. Acidify the plasma, urine or tissue homogenate by addition of 2M HCl to pH of 3.5. Approximately, 50 ml of HCL will be needed per ml of plasma. Allow to sit at 2 - 8°C for 15 minutes. Centrifuge samples for 2 minutes to remove any precipitate.
2. Prepare the C18 reverse phase column (200 mg) by washing with 10 ml of ethanol followed by 10 ml of deionized water.
3. Apply the sample under a slight positive pressure to obtain a flow rate of about 0.5 ml/minute. Wash the column with 10 ml of water, followed by 10 ml of 15% ethanol, and finally 10 ml hexane. Elute the sample from the column by addition of 10 ml ethyl acetate.
4. If analysis is to be carried out immediately, evaporate samples under a stream of nitrogen. Add at least 250 ml of Reagent Diluent to the dried sample. Vortex well, then allow to sit for five minutes at room temperature. Repeat twice more. If analysis is to be delayed, store samples as eluted ethyl acetate solutions at –80°C until the immunoassay is to be run. Evaporate the organic solvent under a stream of nitrogen prior to running the assay and reconstitute as above.

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LTB_4 ELISA ASSAY PROCEDURE

All standards and samples should be run in duplicate.

1. Pipet 100 µl of Standard Diluent (Reagent Diluent or Tissue Culture Media) into the NSB and the B_0 (0 pg/ml Standard) wells.
2. Pipet 100 µl of Standards #1 through # 5 into the appropriate wells.
3. Pipet 100 µl of the Samples into the appropriate wells.
4. Pipet 50 µl of the Reagent Diluent into the NSB wells.
5. Pipet 50 µl of the blue LTB_4-AP conjugate into each well, except the Total Activity (TA) wells and blank wells.
6. Pipet 50 µl of the yellow LTB_4 antibody into each well, except the Blank, TA and NSB wells.

   NOTE: Every well used should be GREEN in color except the NSB wells which should be BLUE. The Blank and TA wells are empty at this point and have no color.
7. Incubate the plate at room temperature (22 - 25°C) on a plate shaker for 2 hours at ≈500 rpm.
8. Empty the contents of the wells and wash by adding 400 µl of the 1X Wash Buffer to every well. Repeat the wash 2 more times for a total of 3 washes.
9. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
10. Add 5 µl of the blue LTB_4-AP conjugate to the TA wells.
11. Add 200 µl of the Substrate Solution to every well. Seal the plate and incubate at 37°C for 2 hours.
12. Add 50 µl of Stop Solution to every well. This stops the reaction and the plate should be read immediately.
13. Blank the plate reader against the Blank wells, read the optical density at 405 nm preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

LTB_4 Plate Layout

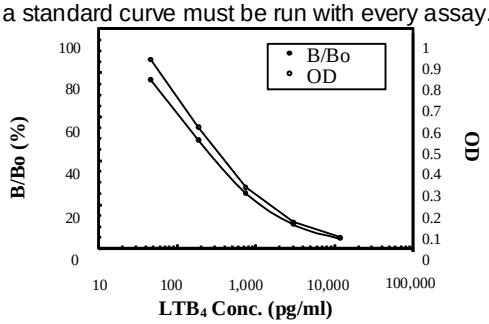
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	Blk	TA	TA	NSB	NSB	Std 1	Std 1	Std 2	Std 2	Std 3	Std 3
B	Std 4	Std 4	Std 5	Std 5	B_0	B_0						
C												
D												
E												
F												
G												
H												

Calculation of Results

Several options are available for the calculation of the concentration of LTB_4 in the samples. It is recommended that the data be analyzed by a 4 parameter logistic curve fitting program. If data reduction software is not available, the concentration of the LTB_4 can be calculated by plotting % Bound [(Net OD/ Net B_0 OD) x 100] versus Concentration of LTB_4 for the standards. Approximate a straight line through the points. The concentration of LTB_4 in the unknowns can be determined by interpolation.

Typical Standard Curve

A typical standard curve is shown below. This curve must not be used to calculate LTB_4 concentrations; a standard curve must be run with every assay.



Typical Quality Control Parameters

Total Activity Added	1.281 x 10 = 12.81
% NSB	0.0%
%Bo/TA	4.36%
Quality of Fit	0.9998 (from 4 parameter fit)
20% Intercept	2,775 pg/ml
50% Intercept	401 pg/ml
80% Intercept	76 pg/ml

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PERFORMANCE CHARACTERISTICS

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols.

Sensitivity

Sensitivity was calculated by determining the average OD bound for 16 wells run as B_0, and comparing to the average OD for 16 wells run with Standard #5. The detection limit was determined as the concentration of LTB_4 measured at 2 standard deviations from the zero along the standard curve.

Sensitivity = 19.4 pg/ml

Linearity

A sample containing 7,990 pg/ml LTB_4 was diluted 6 times 1:2 in the kit Reagent Buffer and measured in the assay. The data was plotted graphically as actual LTB_4 concentration versus measured LTB_4 concentration.

The line obtained had a slope of 0.929 and a correlation coefficient of 0.998.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of LTB_4 and running these samples multiple times (n=16) in the same assay. Inter-assay precision was determined by measuring them in multiple assays (n=8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of LTB_4 determined in these assays as calculated by a 4 parameter logistic curve fitting program.

Intra-assay	LTB_4 (pg/ml)	%CV
Low	305	6.0
Medium	607	6.8
High	1,078	5.9

Inter-assay	LTB_4 (pg/ml)	%CV
Low	99	15.7
Medium	308	16.5
High	507	5.0

Cross-Reactivities

The cross-reactivities for a number of related compounds were determined by dissolving the cross reactant in Reagent Diluent at concentrations from 40,000 to 0.4 pg/ml. These samples were then measured in the LTB_4 assay and the measured LTB_4 concentration at 50% B/B_0 calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

Compound	*Cross Reactivity*
LTB_4	100%
6-trans-12-epi- LTB_4	5.50%
6-trans- LTB_4	4.90%
12-epi- LTB_4	0.94%
PGE_2,$PGF_{2\hat{a}}$, 20-OH- LTB_4, 20-COOH- LTB_4, LTC_4, LTD_4, LTE_4, 5(S)-HETE, 12(S)-HETE, 15(S)-HETE	<0.2%

Sample Recoveries

LTB_4 concentrations were measured in a variety of different samples including tissue culture media, human saliva and urine, and porcine plasma. For samples in tissue culture media, ensure that the standards have been diluted into the same media. LTB_4 was spiked into the undiluted samples of these media, which were then diluted with the appropriate kit Reagent Diluent and then assayed in the kit. The following results were obtained:

Sample	% Recovery	Recommended Dilution
Tissue Culture Media	97.3	None
Human Saliva	114.1	≥1:4
Human Urine	96.9	None
Porcine EDTA Plasma	109.6	1:2 - 1:4

Definition of Key Terms

Total Activity (TA): total enzymatic activity of the LTB_4-AP.
NSB (Non-Specific Binding): non-immunological binding of the LTB_4-AP in the well.
B_0 (Maximum Binding): maximum amount of the LTB_4-AP that the antibody can bind in the absence of free LTB_4.
$\%B/B_0$ (% Bound/Maximum Bound): ratio of the absorbance of a particular sample or standard well to that of the maximum binding (B_0) well.
Standard Curve: a plot of the $\%B/B_0$ values versus concentration of a series of wells containing various known amounts of analyte.

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Quality Control Signature

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