Product Manual

LTB4 ELISA kit

Catalog No. ADI-901-068

480 Well (5 by 96 Well) Kit

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Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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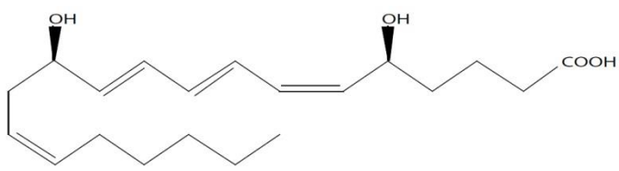
DESCRIPTION

The LTB_4 ELISA kit is a competitive immunoassay for the quantitative determination of LTB_4 in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a polyclonal antibody to LTB_4 to bind, in a competitive manner, the LTB_4 in the sample or an alkaline phosphatase molecule which has LTB_4 covalently attached to it. After incubations at room temperature the excess reagents are washed away and substrate is added. After a 2-hour incubation, the enzyme reaction is stopped and the yellow color generated is read on a microplate reader at 405 nm. The intensity of the bound yellow color is inversely proportional to the concentration of LTB_4 in either standards or samples. The measured optical density is used to calculate the concentration of LTB_4. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

INTRODUCTION

Leukotriene B_4 (LTB_4) is a major product of arachidonic acid metabolism and is formed via the 5-lipoxygenase pathway [3-5]. LTB_4 stimulates leukocyte functions including lysosomal enzyme release[6], adhesion[7,8], and aggregation of polymorphonuclear leukocytes[9]. LTB_4 has been implicated as a potent mediator of inflammatory diseases[10-13] and immunoregulation[14].

LTB_4



PRECAUTIONS

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

1. Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up.
2. Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use.
3. The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions. The activity of the conjugate is affected by concentrations of chelators (>10mM) such as EDTA and EGTA.

4. We test this kit's performance with a variety of samples; however it is possible that high levels of interfering substances may cause variation in assay results.
5. The Leukotriene B_4 Standard provided, Catalog No. 80-0623, is supplied in ethanolic buffer at a pH optimized to maintain LTB_4 integrity. Care should be taken in handling this material because of the known and unknown effects of LTB_4.

MATERIALS SUPPLIED

1. **Goat anti-Rabbit IgG Microtiter Plate, Five Plates of 96 Wells, Catalog No. 80-0060**

   A plate using break-apart strips coated with goat antibody specific to rabbit IgG.

2. **LTB_4 ELISA Conjugate, 28 mL, Catalog No. 80-0102**

   A blue solution of alkaline phosphatase conjugated with LTB_4.

3. **LTB_4 ELISA Antibody, 25 mL, Catalog No. 80-0624**

   A yellow solution of a rabbit polyclonal antibody to LTB_4.

4. **Assay Buffer Concentrate, 27 mL, Catalog No. 80-0011**

   Tris buffered saline, containing proteins and detergents and sodium azide as preservative.

5. **Wash Buffer Concentrate, 100 mL, Catalog No. 80-1287**

   Tris buffered saline containing detergents.

6. **Leukotriene B_4 Standard, 3 x 0.5 mL, Catalog No. 80-0623**

   A solution of 120,000 pg/mL LTB_4.

7. **pNpp Substrate, 100 mL, Catalog No. 80-0076**

   A solution of p-nitrophenylphosphate in buffer. Ready to use.

8. **Stop Solution, 27 mL, Catalog No. 80-0248**

   A solution of trisodium phosphate in water. Keep tightly capped. Caution: **Caustic.**

9. **LTB_4 Assay Layout Sheet, 1 each, Catalog No. 30-0115**
10. **Plate Sealer, 5 each, Catalog No. 30-0012**

STORAGE

All components of this kit, **except the conjugate**, are stable at 4°C until the kit's expiration date. The conjugate **must** be stored frozen at -20°C.

MATERIALS NEEDED BUT NOT SUPPLIED

1. Deionized or distilled water.
2. Precision pipets for volumes between 5 µL and 1,000 µL.
3. Repeater pipets for dispensing 50 µL and 200 µL.
4. Disposable beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. A 37°C incubator.
9. Microplate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.

SAMPLE HANDLING

The LTB_4 ELISA kit is compatible with LTB_4 samples in a wide range of matrices. Samples diluted sufficiently into Assay Buffer can be read directly from the standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions. However, the end user **must verify** that the recommended dilutions are appropriate for their samples. **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 read in the assay, provided the standards have been diluted into the tissue culture media instead of Assay Buffer. There will be a small change in binding associated with running the standards and samples in media. Users should only use standard curves generated in media or buffer to calculate concentrations of LTB_4 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 either the tissue homogenate or urine and plasma samples.

Some samples normally have very low levels of LTB_4 present and extraction may be necessary for accurate measurement. A suitable extraction procedure is outlined below:

Materials Needed

1. LTB_4 Standard to allow extraction efficiency to be accurately determined.
2. 2M hydrochloric acid, deionized water, ethanol, hexane and ethyl acetate.
3. 200 mg C_{18} Reverse Phase Extraction Columns.

Procedure

1. Acidify the plasma, urine or tissue homogenate by addition of 2M HCl to pH of 3.5. Approximately 50 µL of HCl will be needed perml of plasma. Allow to sit at 4°C for 15 minutes. Centrifuge samples in a microcentrifuge for 2 minutes to remove any precipitate.
2. Prepare the C_{18} reverse phase column 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 10ml ethyl acetate.
4. If analysis is to be carried out immediately, evaporate samples under a stream of nitrogen. Add at least 250 µL of Assay Buffer to the dried samples. Vortex well then allow to sit for five minutes in room temperature. Repeat twice more. If analysis is to be delayed, store samples as the 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 assay and reconstitute as above.

Please refer to references 15-18 for details of extraction protocols.

PROCEDURAL NOTES

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided.
8. **Care must be taken to minimize contamination by endogenous alkaline phosphatase.** Contaminating alkaline phosphatase 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.
9. **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.**

REAGENT PREPARATION

1. **Assay Buffer**

   Just before use, prepare the assay buffer by diluting 10 mL of the supplied Assay Buffer Concentrate with 90 mL of deionized water. Discard unused buffer or add up to 0.09% sodium azide (w/v) for storage.

2. **LTB_4 Standard**

   Allow the 120,000 pg/mL LTB_4 standard solution to warm to room temperature. Label five 12x75 mm glass tubes #1 through #5. Pipet 975 µL of standard diluent (Assay Buffer or Tissue Culture Media) into tube #1. Pipet 750 µL of standard diluent into tubes #2 - #5. Add 25 µL of the 120,000 pg/mL standard to tube #1. Vortex thoroughly. Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Add 250 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 and #5.

   The concentration of LTB_4 in tubes #1 through #5 will be 3,000, 750, 188, 46.9 and 11.7 pg/mL respectively. See LTB_4 Assay Layout Sheet for dilution details.

   Diluted standards should be used within 60 minutes of preparation.

3. **LTB_4 Conjugate**

   Allow the conjugate to warm to room temperature. Any unused conjugate should be aliquoted and re-frozen at or below -20°C. Avoid repeated free**ze-thaws of the aliquots.**

4. **Wash Buffer**

   Prepare the Wash Buffer by diluting 5 mL of the supplied concentrate with 95ml of deionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

ASSAY PROCEDURE

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4°C.
2. Pipet 100 µL of standard diluent (Assay Buffer or Tissue Culture Media) into the NSB and the Bo (0pg/ml Standard) wells.
3. Pipet 100 µL of Standards #1 through #5 into the appropriate wells.
4. Pipet 100 µL of the Samples into the appropriate wells.
5. Pipet 50 µL of Assay Buffer into the NSB wells.
6. Pipet 50 µL of blue Conjugate into each well, except the Total Activity (TA) and Blank wells.
7. Pipet 50 µL of yellow Antibody into each well, except the Blank, TA and NSB wells.

   NOTE: Every well 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.
8. Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm.
9. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 2 more times for a total of 3 Washes.
10. 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.
11. Add 5 µL of the blue Conjugate to the TA wells.
12. Add 200 µL of the pNpp Substrate solution to every well. Seal the plate and incubate at 37°C for 2 hours.
13. Add 50 µL of Stop Solution to every well. This stops the reaction and the plate should be read immediately.
14. 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.

CALCULATION OF RESULTS

Several options are available for the calculation of the concentration of LTB_4 in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of LTB_4 can be calculated as follows:

1. Calculate the average Net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

   Average Net OD = Average Bound OD - Average NSB OD

2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

$$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net Bo OD}} \times 100$$

3. Plot Percent Bound 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.

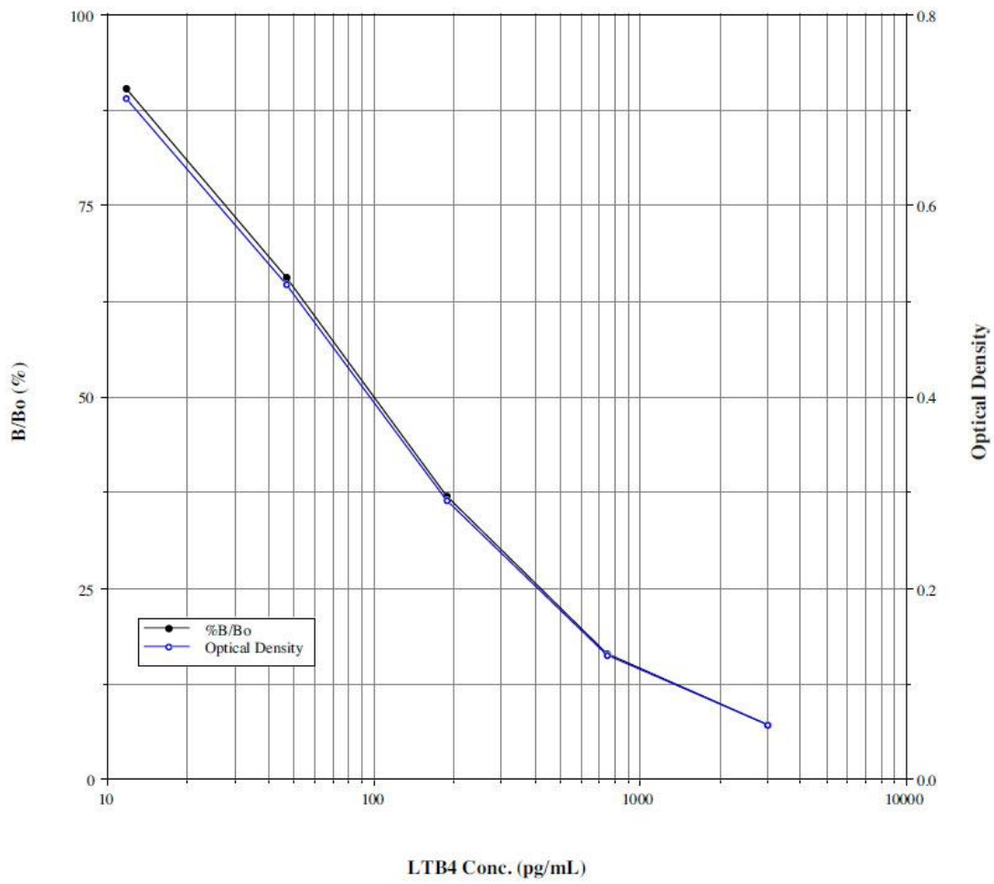
TYPICAL RESULTS

The results shown below are for illustration only and **should not** be used to calculate results.

Sample	Mean OD (-Blank)	Average Net OD	Percent Bound	**LTB_4 (pg/mL)**
Blank OD	(0.145)			
TA	1.466			
NSB	-0.002	0.000		
Bo	0.786	0.788	100%	**0**
S1	0.054	0.056	7.0%	**3,000**
S2	0.127	0.129	16.4%	**750**
S3	0.289	0.291	36.9%	**188**
S4	0.515	0.517	65.6%	**46.9**
S5	0.710	0.712	90.4%	**11.7**
Unknown 1	0.465	0.467	59.3%	**63.5**
Unknown 2	0.155	0.157	19.9%	**556**

TYPICAL STANDARD CURVE

A typical standard curve is shown below. This curve **must not** be used to calculate LTB_4 concentrations; each user must run a standard curve for each assay.



TYPICAL QUALITY CONTROL PARAMETERS

Total Activity Added	=	1.466 x 10 = 14.66
%NSB	=	0.0%
%Bo/TA	=	5.37%
Quality of Fit logistic curve fit)	=	1.000 (Calculated from 4 parameter
20% Intercept	=	552 pg/mL
50% Intercept	=	98 pg/mL
80% Intercept	=	23 pg/mL

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[19].

Sensitivity

Sensitivity was calculated by determining the average optical density bound for twenty (20) wells run as Bo, and comparing to the average optical density for twenty (20) wells run with Standard #5. The detection limit was determined as the concentration of LTB_4 measured at two (2) standard deviations from the zero along the standard curve.

Average Optical Density for the Bo = 0.802 ± 0.025 (3.2%)

Average Optical Density for Standard #5 = 0.696 ± 0.011 (1.6%)

Delta Optical Density (0-11.7 pg/mL) = 0.106

2 SD's of the Zero Standard = 2 x 0.025 = 0.051

$$\text{Sensitivity} = \frac{0.051}{0.106} \times 11.7 \text{ pg/mL} = \textbf{5.63 pg/mL}$$

Linearity

A sample containing 1,000 pg/mL LTB_4 was diluted 6 times 1:2 in the kit Assay 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 0.961 and a correlation coefficient of 0.999.

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 three samples with low, medium and high concentrations of LTB_4 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.

LTB_4 (pg/mL)	Intra-assay %CV	Inter-assay %CV
Low	305	6.0
Medium	607	6.8
High	1,078	5.9
Low	99	15.7
Medium	308	16.5
High	507	5.0

Cross Reactivities

The cross reactivities for a number of related compounds was determined by dissolving the cross reactant (purity checked by N.M.R. and other analytical methods) in Assay Buffer 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/Bo 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	<0.2%
$PGF_{2\alpha}$	<0.2%
20-OH-LTB_4	<0.2%
20-COOH-LTB_4	<0.2%
LTC_4	<0.2%
LTD_4	<0.2%
LTE_4	<0.2%
5(S)-HETE	<0.2%
12(S)-HETE	<0.2%
15(S)-HETE	<0.2%

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling and Standard preparation.

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 kit Assay Buffer 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

* See Sample Handling instructions on page 4 for details.

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NOTES

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