

Enzyme Immunoassay for Leukotriene B₄

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INTRODUCTION

Leukotriene B₄ (LTB₄) is derived from Leukotriene A₄ (LTA₄), which in turn, is biosynthesized from arachidonic acid. LTB₄ is produced in phagocytic cells such as leukocytes and macrophages. It is the most potent chemotactic substance ever described for leukocytes. It also stimulates vascular permeability. It may play a significant role in mediating inflammatory responses. The availability of an EIA for LTB₄ will facilitate the evaluation of the physiological and pathological roles of this lipid mediator.

PRINCIPLES OF PROCEDURE

This is an ELISA (Enzyme-Linked Immunosorbent Assay) for the quantitative analysis of Leukotriene B₄ levels in biological fluid. This test kit operates on the basis of competition between the enzyme conjugate and the LTB₄ in the sample for a limited number of binding sites on the antibody coated plate.

The sample or standard solution is first added to the microplate. Next, the diluted enzyme conjugate is added and the mixture is shaken and incubated at room temperature for one hour. During the incubation, competition for binding sites is taking place. The plate is then washed to remove all of the unbound material. The bound enzyme conjugate is detected by the addition of a substrate that generates an optimal color after 30 minutes. Quantitative test results may be obtained by measuring and comparing the absorbance reading of the sample wells against the standard wells with a microplate reader at 650 nm. The extent of color development is inversely proportional to the amount of LTB₄ in the sample or standard. For example, the absence of LTB₄ in the sample will result in a bright blue color, whereas the presence of LTB₄ will result in decreased or no color development.

MATERIALS PROVIDED

Component	Description	Volume	Storage
EIA Buffer	Buffer used to dilute the Conjugate and LTB ₄ Standards.	30 mL	4°C
10x Wash Buffer	Buffer used to wash the plate prior to color development.	20 mL	4°C
TMB Substrate	TMB substrate used for color development.	20 mL	4°C
5x Extraction Buffer	Buffer used to dilute extracted and non-extracted samples.	30 mL	4°C
LTB₄-HRP Conjugate	Lyophilized LTB ₄ horseradish peroxidase conjugate.	2 vials	-20°C
LTB₄ Standard	1 µg/mL LTB ₄ standard solution.	100 µL	4°C
Coated Plate	96-well microplate coated with a rabbit anti- LTB ₄ antibody.	1 plate	4°C

MATERIALS NEEDED BUT NOT PROVIDED

1. Microplate reader with a 450 nm or 650 nm filter
2. Adjustable micropipettes (10 – 1000 µL) and tips
3. Deionized water
4. Plate cover or plastic film
5. Test tubes
6. 1 N HCl (optional)

EXTRACTION MATERIALS

1. 1 N HCl
2. Ethanol
3. C₁₈ Sep-Pak® Light Column (Waters® Corporation #23501)
4. Petroleum Ether
5. Methyl Formate
6. Nitrogen Gas
7. Centrifuge

STORAGE

1. Store the components of this kit at the temperatures specified on the labels.
2. Unopened reagents are stable until the indicated kit expiration date.
3. Concentrated, reconstituted conjugate has a shelf life of at least two weeks when stored properly.
4. Desiccant bag must remain in foil pouch with unused strips. Keep pouch sealed when not in use to maintain a dry environment. Remove excess air before sealing.

WARNINGS AND PRECAUTIONS

1. Use aseptic technique when opening and dispensing reagents.
2. This kit is designed to work properly as provided and instructed. Additions, deletions or substitutions to the procedure or reagents are not recommended, as they may be detrimental to the assay.

PROCEDURAL NOTES

1. Ensure that the conjugate is completely reconstituted before use. Each vial, when reconstituted, provides sufficient reagent to perform 64 wells (8 strips). If more than 64 assays are to be run, reconstitute both vials and pool the reconstituted conjugate.
2. To minimize errors in absorbance measurements due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel prior to inserting into the plate reader.

SAMPLE PREPARATION

1. Urine and tissue culture supernatant can be assayed after diluting them with diluted Extraction Buffer.
2. Plasma and most other mediums will need to be extracted.
3. Dilute specimens may require an extraction to concentrate the LTB₄.

EXTRACTION PROTOCOL

1. Acidify 1 mL of biological fluid to pH 3.5 with 1 N HCl (about 150 μ L for plasma) and vortex.
2. For tissue, homogenize it in ethanol (5 mL/g). Centrifuge the homogenate for five minutes. Collect the supernatant in a clean tube. Dilute 1 mL of the supernatant with 5 mL of deionized water and acidify to pH 3.5 with 1 N HCl.
3. Precondition the C₁₈ Sep-Pak® Light column by washing the column with 2 mL of ethanol followed by 2 mL of deionized water.
4. Apply the above sample into the column and adjust the flow rate to 1 mL per minute. Reducing the flow rate to 0.5 mL per minute may increase extraction efficiencies. Some samples may clog the column. These samples may be diluted 1:5 in deionized water to improve the flow rate.
5. Wash the column with 1 mL of water followed by 1 mL of petroleum ether.
6. The LTB₄ is eluted by 2 mL of methyl formate.
7. Evaporate the methyl formate eluate with a stream of nitrogen gas.

- Resuspend the residue with 1 mL of diluted Extraction Buffer. The residue may be dissolved in less than 1 mL if the concentration is suspected to be low (<0.1 ng per mL).

REAGENT PREPARATION

- 5x Extraction Buffer:** Dilute the appropriate amount to 1x with deionized water prior to use.
- 10x Wash Buffer:** Add 20 mL of 10x Wash Buffer to 180 mL of deionized water prior to use.
- LTB₄-HRP Conjugate:** Reconstitute by adding 75 μ L of deionized water to each vial. Dilute 110 μ L of Conjugate into 5.5 mL total volume of EIA Buffer.

STANDARD CURVE PREPARATION

The LTB₄ Standard is provided as a 1 μ g/mL stock solution. Use the following tables to dilute a set of standard stock solutions and construct an eight-point standard curve.

Table 1: Standard Stock Preparation

Standard	LTB ₄ Conc. (ng/mL)	Vol. of EIA Buffer (μ L)	Transfer Vol. (μ L)	Final Vol. (μ L)
A	1000	-	Provided	80
B	20	980	20 μ L of A	800
C	2	1800	200 μ L of B	1800
D	0.2	1800	200 μ L of C	2000

Table 2: Standard Curve Preparation

Standard	LTB ₄ Conc. (ng/mL)	Vol. of EIA Buffer (μ L)	Vol. of Stock B (μ L)	Vol. of Stock C (μ L)	Vol. of Stock D (μ L)
S ₀	0	1000	-	-	-
S ₁	0.04	800	-	-	200
S ₂	0.1	500	-	-	500
S ₃	0.2	-	-	-	1000
S ₄	0.4	800	-	200	-
S ₅	1.0	500	-	500	-
S ₆	2.0	-	-	1000	-
S ₇	4.0	800	200	-	-

ASSAY PROCEDURE

- Add 50 μ L of Standards or Samples (may require diluting) to the corresponding wells on the microplate in duplicate. See **Scheme I** for a sample plate layout.
- Add 50 μ L of diluted LTB₄-HRP Conjugate to each well. Incubate at room temperature for one hour.
- Wash the plate three times with 300 μ L of diluted Wash Buffer per well. Wash 5 times if using an automated plate washer.
- Add 150 μ L of TMB Substrate to each well. Incubate at room temperature for 30 minutes.
- Read the plate at 650 nm.

Alternately, the color reaction can be stopped after 10-15 minutes by adding 50 μ L of 1 N HCl and read at 450 nm.

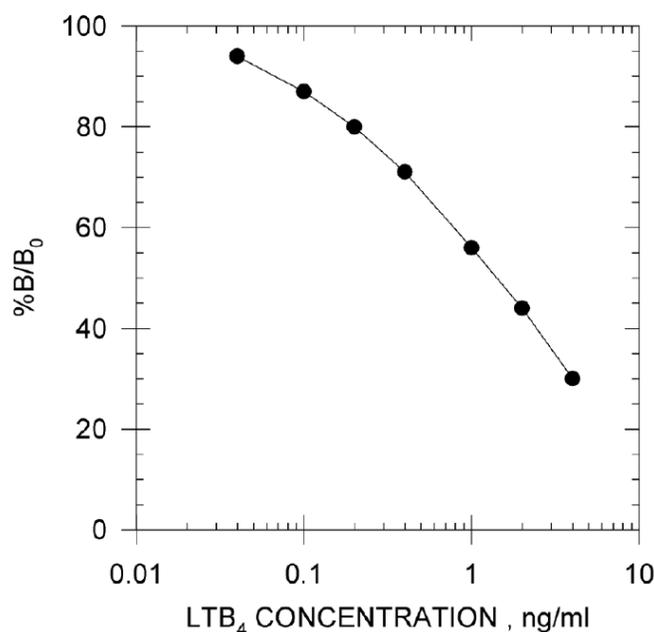
NOTE: If accounting for substrate background, use 2 wells as blanks (BLK) with only 150 μ L TMB Substrate in the wells. Subtract the average of these absorbance values from the absorbance values of the wells being assayed.

Scheme I: Sample Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	S ₀	S ₀	U ₁	U ₁	U ₉	U ₉	U ₁₇	U ₁₇	U ₂₅	U ₂₅	U ₃₃	U ₃₃
B	S ₁	S ₁	U ₂	U ₂	U ₁₀	U ₁₀	U ₁₈	U ₁₈	U ₂₆	U ₂₆	U ₃₄	U ₃₄
C	S ₂	S ₂	U ₃	U ₃	U ₁₁	U ₁₁	U ₁₉	U ₁₉	U ₂₇	U ₂₇	U ₃₅	U ₃₅
D	S ₃	S ₃	U ₄	U ₄	U ₁₂	U ₁₂	U ₂₀	U ₂₀	U ₂₈	U ₂₈	U ₃₆	U ₃₆
E	S ₄	S ₄	U ₅	U ₅	U ₁₃	U ₁₃	U ₂₁	U ₂₁	U ₂₉	U ₂₉	U ₃₇	U ₃₇
F	S ₅	S ₅	U ₆	U ₆	U ₁₄	U ₁₄	U ₂₂	U ₂₂	U ₃₀	U ₃₀	U ₃₈	U ₃₈
G	S ₆	S ₆	U ₇	U ₇	U ₁₅	U ₁₅	U ₂₃	U ₂₃	U ₃₁	U ₃₁	U ₃₉	U ₃₉
H	S ₇	S ₇	U ₈	U ₈	U ₁₆	U ₁₆	U ₂₄	U ₂₄	U ₃₂	U ₃₂	BLK	BLK

CALCULATIONS

1. After the substrate background has been subtracted from all absorbance values, average all of your duplicate well absorbance values.
2. The average of your two S₀ values is now your B₀ value. (S₁ now becomes B₁, etc.)
3. Next, find the percent of maximal binding (%B/B₀ value). To do this, divide the averages of each standard absorbance value (now known as B₁ through B₇) by the B₀ absorbance value and multiply by 100 to achieve percentages.
4. Graph your standard curve by plotting the %B/B₀ for each standard concentration on the y-axis against concentration on the x-axis. Draw a curve by using a curve-fitting routine (i.e. 4-parameter or linear regression).
5. Divide the averages of each sample absorbance value by the B₀ value and multiply by 100 to achieve percentages.
6. Using the standard curve, the concentration of each sample can be determined by comparing the %B/B₀ of each sample to the corresponding concentration of standard.
7. If the samples were diluted, the concentration determined from the standard curve must be multiplied by the dilution factor.

Figure 1: Typical Standard Curve

CROSS REACTIVITY

Leukotriene B ₄	100.0%	12(S)HETE	<0.10%
6-trans-Leukotriene B ₄	25.00%	12(R)HETE	<0.10%
Leukotriene B ₅	14.58%	15-HETE	<0.01%
5(S)12(S)di-HETE	6.00%	Prostaglandin A ₂	<0.01%
Leukotriene D ₄	0.96%	Prostaglandin B ₂	<0.01%
20-hydroxy-Leukotriene B ₄	0.50%	Prostaglandin D ₂	<0.01%
Leukotriene E ₄	0.30%	Prostaglandin E ₂	<0.01%
Leukotriene C ₄	0.20%	Prostaglandin F _{2α}	<0.01%
5(S)HETE	0.15%	6-keto-Prostaglandin F _{1α}	<0.01%
20-carboxy-Leukotriene B ₄	<0.10%	Thromboxane B ₂	<0.01%
Arachidonic Acid	<0.10%		

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